REGULATION OF HEPATIC ALS AND IGFBP-1 EXPRESSION

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ABSTRACT

The insulin-like growth factor (IGF) system is composed of IGF, IGF binding proteins (IGFBP-1 to -10) and the acid labile subunit (ALS). IGF exists as two isoforms, IGF-I and IGF-II. IGF-I is the major circulatory form and is primarily secreted by the liver. It functions to regulate proliferation and differentiation in a number of different cell types and elicits an insulin-like metabolic effect. As well as being regulated at levels of transcription and translation, IGF-I activities are also regulated through formation of complexes in circulation. IGF complexes form as binary complexes, such as the IGFBP-1 complex, and ternary complexes containing IGF-I, IGFBP-3 and ALS. Binary and ternary IGF complexes function to maintain stable pools of bioactive IGF-I. They also function to increase IGF half-life and sequester IGF in the bloodstream.

ALS and IGFBP-1 are well characterized and exist as 85 kDa and 32 kDa proteins, respectively. They are expressed primarily in liver hepatocytes. Circulating ALS binds the IGF-I-IGFBP-3 complex and increases IGF half-life from 10 min in the IGFBP-3 binary complex to 10-15 hr in the ternary complex. IGFBP-1 binds IGF-I and increases the half-life from 10 min to 30 min. The ternary complex is the predominant IGF-I binding protein complex found in circulation. The IGFBP-1 complex represents only a small fraction of circulating IGF complexes.

In this thesis ALS and IGFBP-1 regulation were investigated in terms of expression related to metabolic modulators and streptozotocin (STZ)-induced diabetes. Results from rat studies showed a decreased liver ALS gene expression in STZ-induced diabetic rats. STZ-treatment in rats mimics type-I diabetes with no change in secreted insulin with increase of circulatory glucose. The administration of insulin into the STZ-induced diabetic rats brought ALS levels to that of the untreated controls. ALS expression was positively regulated by insulin in H4IIE hepatoma cells. Growth hormone (GH), glucose, dexamethasone also positively regulated ALS gene expression while cAMP (2-b-cAMP) acted as a negative regulator in H4IIE cells. HepG2 cells expressing constitutively active protein kinase B (PKB) (HepG2-PKB-CA) increased ALS gene expression to levels 20% higher then parental HepG2. Insulin treatment of these cells

unexpectedly increased ALS levels in both parental and PKB-CA HepG2. This may have indicated a partial regulatory role of the mitogen activated protein (MAP) kinase pathway as PKB was thought to be over-expressed therefore rendering the insulin signal redundant. Inhibition of the phosphoinositol-3 (PI-3) kinase and MAP kinase pathways through wortmannin and PD98059 incubation, respectively, suggested a possible interplay or crosstalk between the two pathways in insulin signaling. PKB is known to be activated through the PI-3 kinase pathway. Results suggested possibility that PKB may interact through the MAP kinase pathway in regulation of ALS gene expression. The activity of cAMP on ALS gene expression may occur through interaction with the PI-3 kinase pathway as inhibition enhanced the negative effect of cAMP on ALS expression.

The secretion of IGFBP-1 was positively regulated by glucose and GH and negatively regulated by insulin in H4IIE cells. HepG2-PKB-CA cells showed significantly lower IGFBP-1 secretion as compared to parental HepG2 cells. The involvement of the PI-3 and MAP kinase pathways in the modulator-mediated effect on IGFBP-1 secretion were. As observed for ALS expression, the effect of insulin on IGFBP-1 secretion may also be affected through interplay or crosstalk between the PI-3 kinase and MAP kinase pathways. Glucose and GH effected IGFBP-1 expression and secretion independent of these pathways although glucose expression may interact in some way through the PI-3 kinase pathway. Our investigation of hepatic regulation of IGFBP-1 secretion and ALS gene expression has shown regulatory roles for the metabolic hormones tested, especially insulin. Mechanisms of cell signaling have also been approached with the use of pathway inhibiters and HepG2-PKB-CA cells. Much work is yet to be done to fully understand the effects of insulin and other hormones on the secretion and expression of IGFBP-1 and ALS.

This work is dedicated to the memory of my father

Frank Hepp (1946-2000)

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LIST OF ABBREVIATIONS

ALS	acid labile subunit
AP-2	Adaptor protein- 2
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CREB	cAMP response element binding protein
DDH_2O	Double distilled H_2O
DEPC	Diethyl pyrocarbonate
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetate
ERK-1	Externally regulated kinase-1
FKHR	Fork head related protein
G-6-P	Glucose-6-phosphate
GH	Growth hormone
GC	Glucocorticoid
GR	Glucocorticoid receptor
GDP	Guanidine diphosphate
GTP	Guanidine triphosphate
HNF	Hepatocyte nuclear factor
IGF-I	Insulin-like growth factor -1
IGF-II	insulin-like growth factor -2
IGF-IR	insulin-like growth factor -1 receptor
IGFBP-1	IGF binding protein -1
IGFBP-3	IGF binding protein -3
IRE	Insulin response element
IRS-1	Insulin receptor substrate -1
IR	Insulin receptor
Jak2	Janus kinase 2
LB	Luria broth
LRR	Leucine-rich repeat
L-PK	L-type pyruvate kinase
MAP	Mitogen activated protein
MEK	MAP/Erk Kinase
MOPS	Morpholino propane sulfonic acid
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rampamycin
$p70^{s6k}$	p70 s-6 kinase
PDK-1	Phospho dependant kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PEST	Pro-Glu-Ser-Thr motif
PI-3K	Phosphoinositol -3 kinase
PI-3,4,5-P_3	Phosphoinositol-3, 4, 5-triphosphates
PKA	Protein kinase A
PKB	Protein kinase B

pY	Phosphotyrosine
RGD	Arg-Gly-Asp motif
RI	Ribonuclease inhibitor
RT	Room temperature
RPPO	Ribosomal phosphoprotein PO
SDS	Sodium dodecyl sulfate
SSC	Standard Saline Citrate
STZ	Streptozotocin
STAT5	Signal transducer and activator of transcription-5
T_3	Triiodo-L-thyronine
TE	Tris-EDTA (buffer)
TF	Transcription factors
UTR	Untranslated region

1.0 INTRODUCTION

Cell regulation occurs at transcriptional and post-transcriptional levels through covalent and non-covalent modifications. Therefore, the regulation of protein activity involves many factors and many levels of function. The complexity of protein function increases when one looks from the cell to the whole animal. The regulation of secreted proteins occurs either through direct modification of the protein, itself, or it can be modulated through binding to specific binding proteins in circulation. The latter effect may be to sequester the protein in the bloodstream, making it unavailable for binding to target tissues, or it may be to increase the stability of the protein in circulation allowing a larger pool of bioactive protein.

The insulin-like growth factors (IGF-I and IGF-II) are synthesized by most tissues in the body and are abundant in circulation. They function to regulate proliferation and differentiation in a number of different cell types and elicit an insulin-like metabolic effect. IGFs exert their effects at the cellular level through their binding to IGF receptors (IGF-IR). IGF also binds the insulin receptor as well as IGF/insulin hybrid receptors but binds these alternative receptors with a much lower affinity than for the IGF-IR (Werner, 1999). In circulation, IGF-I is primarily in a complex consisting of IGF-I, IGF-I binding proteins (IGFBPs) and the acid labile subunit (ALS). Ten IGFBPs and ALS combine to comprise the IGFBP complexes. IGFBPs, with the exception of IGFBP-3 and -5, form binary complexes that account for small percentages of the total complexes formed in circulation. The most abundant complex found is the ternary complex formed by IGF-I, IGFBP-3 and ALS.

Both, IGFBP-1 and ALS have been highly characterized. IGFBP-1 is a 32 kDa protein synthesized primarily in the liver and kidney and ALS is an 85 kDa glycoprotein primarily secreted by liver. IGFBP-1 both inhibits and potentiates the metabolic and mitogenic effects of IGF-I. Addition of purified IGFBP-1 inhibits binding of IGFs to cell surfaces but pre-incubation with IGFBP-1 results in a potentiation of IGF-1 activity (Elgin *et al.,* 1987; Lewitt *et al.,* 1991). IGFBP-1 has a 5-fold greater affinity for IGF-I than the IGF-IR and may be important in the transport of IGF-I across the endothelial barrier. IGF-1 in the IGFBP-1 complex extends the IGF-I half-life from 10 min to 30 min

(Jones *et al*., 1990). ALS, like IGFBP-1, also extends the IGF-I half-life, but in the ternary complex IGF-I half-life is extended to 10-15 hr (Baxter *et al.,* 1989). The ternary complex disables any transport of IGF-1 across endothelial barriers and therefore sequesters the IGF-I in circulation. If ALS is not bound to the IGFBP-3, the IGFBP-3-IGF-I complex acts much like the IGFBP-1 complex.

Hepatocyte cells are sensitive to hormone levels in respect to changes in metabolic gene expression due to downstream cell signaling. IGFBP-1 and ALS act to regulate the IGF bioactivity including the IGF mediated effects on metabolism. Metabolic modulator hormones such as insulin, dexamethasone and glucagon therefore should have effects on ALS and IGFBP-1 expression. Glucose has also been shown to possibly regulate cell expression and may also regulate ALS and IGFBP-1 expression (Snyder *et al*., 1990). GH promotes IGF expression and thus IGF activity (Norstedt *et al*., 1987). It is hypothesized that GH will also promote ALS and IGFBP-1 expression in order to stabilize IGF in circulation and thus promote IGF effects on responsive tissues. Somewhat aside, it is hypothesized that H4IIE cell ALS expression will be regulated as has been shown in primary hepatocytes. ALS gene regulation has not been investigated in H4IIE or HepG2 cells due to instability of the ALS mRNA transcript. Newly available rALS probes may allow the possibility of successful visualization and measurement of H4IIE and HepG2 ALS gene expression through Northern analysis.

As IGF ternary complex formation with IGFBPs and ALS may regulate IGF-I activity, it is of interest to investigate the regulation of ALS expression in the liver. Investigation of liver cell ALS gene expression will give a clearer picture of the role of hormonal regulation of IGF and may allow conclusions to be made as to how the IGF system changes as result of changes in hormone levels such as in the case of altered insulin levels during diabetes. Primary hepatocytes have shown insulin to positively regulate ALS expression (Dai *et al.,* 1994a). Streptozotocin-treated rats have decreased insulin levels and therefore should show a decrease in ALS levels. In addition, it has been suggested that IGF-I may have a role in tumour development related to diabetes. The altered nutritional and hormonal states of diabetes results in changes in the expression of ALS altering the availability of IGF-I. Although this idea is not supported through the

results of this thesis it is nonetheless a strong relationship which should be investigated in the near future.

The regulation of IGFBP-1 secretion and ALS gene expression by hormones is primarily through cell signaling mechanisms which alter expression. IGFBP-1 secretion may also be controlled by posttranslational modifications such as phosphorylation which has been shown in literature and may involve similar signaling. Regulation of expression through insulin is mediated through two primary signaling pathways, the MAP kinase and the PI-3 kinase pathways. IGFBP-1 has been shown to be regulated through the PI-3 kinase pathway through activation of PKB and downstream effectors such as mTOR and FKHR proteins (Durham *et al.,* 1999; Patel *et al.*, 2002). Other studies have shown involvement of the MAP kinase pathway (Band *et al.,* 1997). I hypothesize that although PKB may be involved the regulation of IGFBP-1 this may occur through crosstalk between PKB, the PI-3 kinase pathway and the MAP kinase pathway. It was also of interest to investigate whether glucose and GH may interact with the PI-3 kinase and MAP kinase pathways in IGFBP-1 regulation due to the potential involvement of many of the same effecter molecules. In the case of ALS regulation by insulin it is hypothesized that this regulation is through the PI-3 kinase pathway and PKB activation. As no data exists for this to date this idea is only supported by the fact that insulin has been shown to positively regulate ALS gene expression (Dai *et al.,* 1994a). As cAMP has been shown to be a negative regulator of ALS expression I hypothesize that this altered regulation may be through the involvement of effecter molecules downstream of the cAMP signal altering an activity of the PI-3 kinase pathway on ALS expression.

2.0 REVIEW OF LITERATURE

2.1 Overview on Metabolic Role of IGF in Regulation

2.1.1 Animal Tissue Growth and Regulation

IGF regulation of animal tissue growth and differentiation occurs throughout animal development. During embryonic and post-embryonic development IGF-I and IGF-II are required for normal growth and differentiation. IGFBPs, ALS and IGFBP proteases also function in growth and differentiation through the regulation of IGF activity and in some cases may act independent of IGF. IGF-I and IGF-II are small peptides of approximately 7 kDa and are highly similar in sequence and conformation. The small differences in sequence may reflect in their receptor specificities. IGF-I binds to the IGF-I receptor (IGF-IR) whereas IGF-II binds to the mannose 6-phosphate receptor (M-6-P receptor or IGF-IIR). IGF-II may also bind IGF-IR and signal through this receptor (Nakae *et al.,* 2001). The downstream effects of the M-6-P receptor activation by IGF-II are unclear but have been reported to have roles in fetal organogenesis, lysosomal enzyme trafficking, cytotoxic T cell-induced apoptosis and tumour suppression. IGF-II is the major IGF expressed during early embryonic development (Bhaumick *et al.,* 1987). The IGF-II gene is subject to genomic imprinting, a process whereby the gene is expressed primarily by one parental allele. Imprinted genes have been shown to be important in fetal development (Reik *et al.,* 1996, Claire *et al.,* 1996). Experiments utilizing homologous recombination have resulted in IGF-II null mice 60% smaller in size than normal mice, but otherwise normal and fertile (Allan *et al.,* 2001*)*. The resulting phenotype suggests that IGF-II, although important for normal development, may not be essential for survival.

The IGF-IR is highly similar to the insulin receptor (IR) in sequence and function. Both have similar downstream signaling events which include many of the same signaling molecules as seen with the IR such as insulin substrate-1 (IRS-1) (Figure 2.1). Ligand binding analysis in chicken embryos shows IGF-IR/IGF-I association in stages as

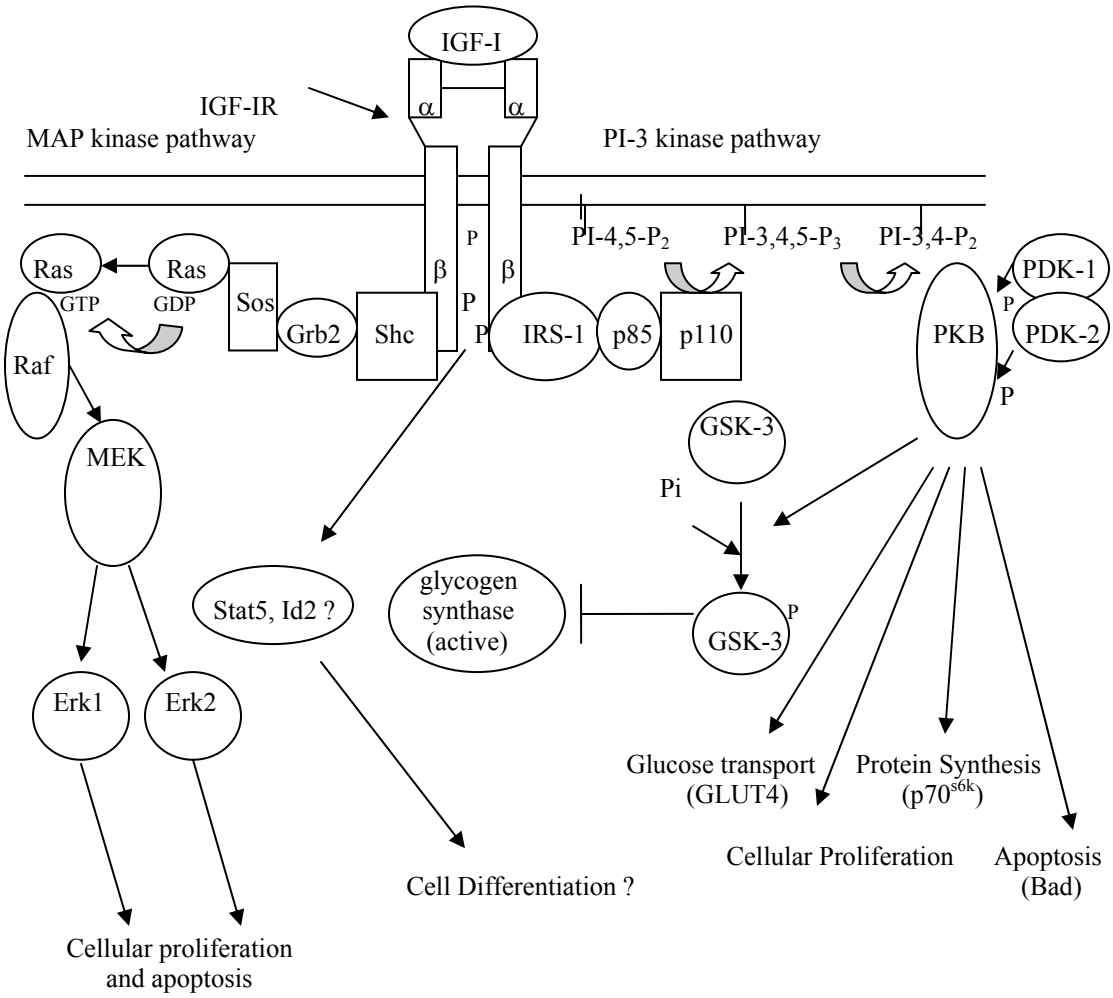


Figure 2.1. Signal transduction mechanism of insulin-like growth factor-I receptor. Once ligand (IGF-I) is bound, IGF-IR is activated leading to phosphorylation events and downstream activities involving multiple proteins. Depicted to the left of the IGF-IR is the mitogen activated protein (MAP) kinase pathway which signals for cell proliferative and apoptotic activities. Depicted to the right of the IGF-IR is the PI-3 kinase pathway. Briefly, IRS-1 is phosphorylated and activated leading to the phosphorylation of phosphoinositols. PKB is activated downstream of PI-3,4-P_2 effecting glucose transport, glycogen synthesis, cellular proliferation, protein synthesis and apoptosis. Also depicted is the activation of molecules such as Stat5 and Id2 which may be involved in cell differentiation.

Abbreviations: P , Phosphorylation; Remaining abbreviations given in the List of Abbreviations on page xii

early as gastrulation and neurulation. In addition, IGF-I dependent tyrosine kinase activity has been detected in day 2 embryos (Claire *et al.,* 1996). IGF-IR mRNA has also been detected in chicken blastoderm by PCR analysis (Bassas *et al.,* 1985). Analysis of growth kinetics in mouse embryos carrying IGF-I null mutations indicate growth deficiencies if heterologous in mutation. If homologous in mutation, a 60% mortality rate occurs along with growth retardation (Baker *et al.,* 1993). In contrast, null mutations in the gene encoding IGF-II do not cause mortality in mice but do result in growth retardation similar to that seen in heterologous IGF-I null mutations. Double knockouts of IGF-I and IGF-IR in mice have resulted in malformation of limbs, muscle dystrophy and a 95% mortality rate at the time of birth (Liu *et al.,* 1993). The growth promoting activity of both IGF-I and IGF-II and their receptors appear to be required for proper growth of tissues and act as cell survival factors. However, IGF-I may also be required for specialized developmental activities required for survival.

The involvement of IGF-I system activity in mammalian and avian limb bud formation has also been reported. Limb bud formation has been shown to involve the cell survival promoting activity of IGF within the rapidly dividing mesoderm tissue proximal to the apical endodermal ridge (AER) region of the developing limb bud. Expression of IGF-I and IGF-IR mRNA has been detected in this region. In IGF-I and IGF-IR knockouts, limb formation is attenuated leading to deformation (Allan *et al.,* 2001). Other components of the IGF axis such as IGFBP-2 and IGFBP-5 have been detected and implicated in the apoptotic activity which is necessary for limb development at later stages (Allan *et al.,* 2001). IGF-I/IGFBP association sequesters IGF and disables the mitogenic response. IGF-I/IGFBP association in developing limb tissue may therefore allow decreased tissue growth and act as an alternative to apoptotic activity in the control of tissue growth. In the developing limb, possible IGF-I independent IGFBP action may also be responsible as recent studies show IGFBP binding to specific receptors and changing expression within certain cell types (Allan *et al.,* 2001).

Cell differentiation is a cellular event where a cell may become terminally programmed for a certain function. The activity of the IGF-IR has been implicated in the induction of terminal differentiation. Under certain conditions, myoblasts, osteocytes, adipocytes, neurons and haemopoietic cells can be induced to differentiate by IGF-I

(Baserga *et al.,* 2001). IGF-IR is known to use IRS-1 as a substrate and cause downstream mitogenic effects. Recent evidence suggests that in the absence of IRS-1 (IRS-1 gene knockout) the activated IGF-IR may signal for cell differentiation. For example, in 32D cells, murine haemopoeitic cells of the myeloid lineage, there is no IRS-1 and IRS-2 expression and a very low IGF-IR expression. In culture, these cells undergo apoptosis within 24 hours following the removal of interleukin-3 (IL-3) from the media. When 32D cells express human IGF-IR cDNA they survive with the absence of IL-3. With the addition of IGF-I they grow for 48 hours and then differentiate along the granulocytic pathway. In 32D cells expressing IRS-1 cDNA this differentiation is inhibited (Valentis *et al.,* 1999). The absence of the IRS-1 substrate leads to a contradictory receptor signaling pathway leading to differentiation. Separate studies investigating mutation of the IGF-IR domains in H19-7 rat neuronal cells have been done to determine the region of the receptor required for differentiation. Tyrosine 950 and serine 1280-1283 residues in the C-terminus of the receptor are required for IGF-I-induced differentiation although they are dispensable for IGF-I-mediated growth (Claire *et al.,* 1996). The involvement of downstream proteins in the IGF-IR differentiation pathway has not yet been clearly determined but possible roles for Id2 and STAT5 proteins have been suggested (Figure 2.1) (Baserga *et al.,* 1999; Valentinis *et al.,* 1999; Navarro *et al.,* 2001,).

Discussion of IGF activity must be accompanied with a discussion on the role of IGF binding proteins, ALS and IGFBP proteases. The role of IGF in cell survival and developmental biology is well documented, but the study of IGFBP, ALS and IGFBP proteases in relation to IGF activities is in its infancy. The IGFBPs and ALS have a counter regulatory effect. Association with IGF-I in the bloodstream sequesters the protein and disables the effects of IGF. The aforementioned 10 IGFBPs have been characterized and will be discussed in some detail later in this section. All IGFBP genes are tightly structurally associated with and are thought to have co-evolved with the HoxA to HoxD genes. The Hox family of proteins are DNA binding proteins which have an important role in embryonic development. The co-evolution may follow in function as it is known that many IGFBPs are expressed in early developmental stages (Allander *et al.,* 1995). IGFBP-2 expression has been shown in rat embryo amongst rapidly dividing or

differentiating tissues. The function of IGFBP-2 in the embryo may be in the modulation of IGF activity or in activities independent of IGF. IGFBP-5 expression in early rat embryo has also been shown. It has been shown that IGFBP-2 and IGFBP-5 have highly complimentary expression patterns that often overlap in adjacent cell populations indicating a complimentary function. Expression of all IGFBPs has been shown by day 14 in the rat embryo (Allan *et al.,* 2001).

Genetic modification has shown the involvement of the IGFBPs during developmental processes. In transgenic mice, the over expression of IGFBP-1 causes mild growth retardation (Rajkumar *et al.,* 1995) while targeted gene disruption of IGFBP-2 showed surprisingly little effect on development probably due to the complementary activity of IGFBP-5. IGFBP-3 over expression showed an increased size of mouse liver, heart and spleen (Murphy *et al.,* 1995). Gene deletion of ALS caused a 40% decrease in IGF-I in circulation and a 10% reduction in body mass due to the assumed destabilization of the IGF binding protein complex and the resulting decrease in IGF-I half-life (Yakar *et al.,* 2002).

Some IGFBPs may have receptor binding activity independent of IGF-I which may affect growth and development. Receptors have been found for IGFBP-1 (Jones *et al.,* 1993), IGFBP-2 (Rauschnabel *et al.,* 1999), IGFBP-3 (Oh *et al.,* 1993) and IGFBP-5 (Andress *et al.,* 1995) but none of these receptors has yet been cloned. IGF independent effects have been found to include modulation of bone cell proliferation (Mohan *et al.,* 1995) as well as growth arrest of breast and prostate cancer cells (Oh *et al.,* 1993; 1995). The recognition of IGFBP protease activity is a recent development and may have a profound effect on the understanding of the regulation of IGF activities including developmental processes. Categories of IGFBP proteases, to date, include kallikriens (Cohen *et al.,* 1992), cathespins (Canover *et al.,* 1994) and matrix metalloproteinase proteins (MMPs or matrixins) (Fowlkes *et al.,* 1994). Proteolysis of IGFBP-2 to 6 has been shown in many clinical states and cellular systems. It has also been shown that IGFBP proteases are important regulators of autocrine and paracrine growth seen in processes such as ovarian follicular growth and atresia (Watterau *et al.,* 1999*)*.

2.1.2 Metabolic Function in Animal Tissue

IGF-I has important effects on carbohydrate, lipid and protein metabolism. The insulin-like effects of IGF-I in cells expressing IGF-IR on the cell surface is likely due to a high similarity between IGF-IR and IR. IGF-IR is expressed in adult animal tissues, excluding the liver, and does not show significant tissue specific expression. It is known that, like the IR, IGF-IR has kinase domains which phosphorylate IRS-1 and lead to downstream signaling pathway activation. Through the Phosphoinositol-3 (PI-3) kinase pathway, protein kinase B (PKB) and Protein phosphatase-1G (PP-1G) are activated and Glycogen synthase kinase-3 (GSK-3) is inactivated which in turn activates glycogen synthase (Syed *et al.,* 2000). Active PKB also leads to the stimulation of $p70^{s6k}$ that also have downstream effects on protein synthesis (Kadowaki *et al.,* 1996). These activated pathways give rise to anti-apoptotic effects, glycogen storage and decreased glucose in circulation (Figure 2.1). The effects of infusions of human recombinant IGF-I (hrIGF-I) in patients have been determined on glucose metabolism. There was a ten-fold increase in glucose consumption with a 30 μg / kg * hr IGF-I dose (Turkalj *et al.,* 1992; Zenobi *et al.,* 1992). Also shown were increased glucagon concentrations with decreasing glucose levels in circulation. Separate studies using subcutaneous injection of IGF-I over a five day period showed no change in glucose turnover, but showed increased insulin sensitivity during a euglycemic/hyperinsulinemic clamp treatment (Hussain *et al.,* 1993).

The effect of hrIGF-I (80% in free form – not bound to IGFBPs) on the production of hypoglycemia in healthy adults is 6% as potent as insulin on a molar basis (Guler *et al.,* 1987). Comparative analysis of the effects of insulin and IGF-I has been well documented. With the use of a euglycemic clamp and IGF-I infusion it was shown that glucose disposal mediated by IGF-I had a slower onset than insulin as well as a slower return to normal glucose levels after IGF-I infusion had ceased (Russel-Jones *et al.,* 1995). Boulware *et al.* (1994) also observed increases in glucose uptake and inhibition of hepatic glucose production in studies with acute IGF-I infusions. Muscle tissue is especially responsive to the IGF-I signal while hepatic tissue response is negligible due to absence of IGF-IR in this tissue. The IGF-I induced response in muscle is likely due to the presence of a higher number of IGF-IR. In the case of hepatic tissue

where IGF-IRs is almost non-existent, its inhibitory effects on hepatic glucose production may occur through alternate receptors. IGF-I may interact with alternative receptors such as the IR or hybrid IGF-I/IR or may affect substrate delivery for hepatic gluconeogenesis. It is of importance to note that in the above studies showing the hypoglycemic effect of IGF-I (Boulware *et al.,* 1994; Russel-Jones *et al.,* 1995) the dosage used was ten-fold higher than insulin and could have also been influenced by dietary states, routes of administration, time and dose.

A consequence of increased IGF-I levels in circulation is a decrease in growth hormone (GH) and insulin release. IGF-I, which is positively regulated by GH and insulin, acts to regulate GH concentrations through a feedback mechanism involving GH releasing pituitary and the growth hormone releasing hormone (GHRH) secreting hypothalmus. Decreased IGF-I, through mice *igf1* gene deletion and low dose euglycemic hrIGF-I infusion, leads to GH hypersecretion (Hartman *et al.,* 1993). GH is known to have an antagonistic effect on insulin action (Dominici *et al.,* 2002) through reducing IR levels (Venkatesan *et al.,* 1995) and IR/IRS-1 phosphorylation (Balbis *et al.,* 1996). Increased GH concentrations, therefore, have an effect on glucose metabolism by limiting insulin mediated glucose removal. GH has been found to be a cause of insulin resistance in rodent models exhibiting chronic GH excess (Yakar *et al.,* 2001). Studies with liver specific *IGF-I* gene inactivation in mice (LI-IGF-I $^{-}$ /$^{-}$ mice) show increased insulin secretion compared to controls over a nine month period. Measurements of plasma insulin before and after intravenous injections of glucose (1 g/kg) showed insulin levels in LI-IGF-I $^{-}$ /$^{-}$ mice were more than 2-fold higher than control mice. With increased insulin concentrations, there was no difference in glucose clearance between the LI-IGF-I $^{-}$ /$^{-}$ mice and the control mice even though insulin levels in the transgenic mice were higher (Isaksson *et al.,* 2001). Decreased liver derived IGF-I, therefore, may lead to changes in metabolism due to increased insulin secretion, but the inability to bring down glucose concentration irrespective of increased insulin secretion may indicate a state of insulin resistance. Separate studies looking at the effects of hrIGF-I administration on insulin levels and insulin secretion showed direct suppression of insulin secretion in human subjects (Zenobi *et al.,* 1992) through a reciprocal mechanism in agreement with the above data. IGF-I has also been shown to interact or interfere with the metabolic

effects of thyroid hormone (TH) in liver cells through the modulation of thyroid hormone receptor (THR) expression and the activity of thyroid responsive enzymes such as α-glycerophosphate dehydrogenase (α–GPD) and malic enzyme (ME) (Pellizas *et al.,* 1998).

The role of IGF-I in the regulation of lipid metabolism is similar to that of insulin. hrIGF-I infusion shows decreased plasma triglyceride, β-hydroxybutyrate and free fatty acid (FFA) levels with increasing IGF-I presence in circulation. Decreased β-hydroxybutyrate may indicate inhibition of ketogenesis by IGF-I, but it is likely due to decreases in plasma FFA (Turkalj *et al.,* 1992). Similar results were obtained with insulin infusion (Kazumi *et al.,* 1986). The involvement of IGF-I in lipolysis and subsequent release of FFA is still debatable. Russel-Jones *et al.* (1995) and Boulware *et al.* (1994) have both shown decreases in non-esterified fatty acids (NEFA) with IGF-I infusion, but Hussain *et al.* (1993) have shown an increase in NEFA with IGF-I subcutaneous injection in healthy patients. Other work has been done using liver specific *igf-I* gene inactivation in mice (LI-IGF-I ¯/¯ mice). There was a 26.5% decrease in fat mass in thirteen month old transgenic mice compared to control (Isaksson *et al.,* 2001). Leptin levels have been associated with the amount of body fat in mice (Ahren *et al.,* 1997). Leptin (from the Greek *leptos*, meaning thin) is a protein hormone which effects the regulation of body weight, metabolism and reproductive function. The protein is encoded by the obese (ob) gene and is mainly expressed in adipocytes. Leptin levels have been positively correlated with obesity due to an increased number of adipocytes in obesity. The injection of recombinant mouse or human leptin into ob-/ob - mice results in a 50% decrease in body weight and a large decrease in food intake. In LI-IGF-I ¯/¯ mice, increases in leptin levels were also reported. Increased leptin levels may also lead to a 26.5% decrease in fat storage as seen in transgenic mice lacking liver derived IGF-I (Isakkson *et al.,* 2001). The fat levels became similar after thirteen months, but the control animals had a higher fat percentage than transgenic mice. Although it has been shown that IGF-I binds to adipocytes its metabolic effects are through the IR (Bolinder *et* al 1987). It has not been yet determined whether insulin has a similar effect on leptin levels. The decrease in liver derived IGF-I expression also caused an increase in cholesterol levels mainly in the high density (HDLP) and low density lipoprotein fractions (LDLP) (Isaksson *et al.,* 2001).

Protein metabolism involves the synthesis and breakdown of protein, the energy consumed and/or released in these processes and the byproducts produced. The *in vivo* metabolic effects of IGF-I are difficult to determine due to the consequential modifications in the levels of other hormones known to affect protein metabolism such as insulin and GH. However, data exists that shows IGF-I to have a similar effect as insulin, but with 14 times less potency (Giordano *et al.,* 1995). The overall effect of IGF-I on protein metabolism has been in reduction of proteolysis and little or no effect on protein synthesis. In human subjects, increased IGF-I dose exhibited a dose-dependent decrease in plasma leucine and α-ketoisocaproate, the ketoacid metabolite of leucine. The decrease in whole body leucine flux and leucine oxidation has also been observed with increase in IGF-I (Turkalj *et al.,* 1992). This indicates a decrease in overall protein breakdown and an inhibition or irreversible loss of leucine due to oxidation catalyzed by the rate limiting branched-chain alpha-keto acid dehydrogenase (BCKD) complex. Similar to insulin, IGF-I may signal cells to limit the usage and breakdown of protein by gluconeogenesis and instead follow metabolic pathways that favor carbohydrate breakdown and usage which is far less expensive in terms of energy costs. Most of the above data was performed on fasted subjects where amino acid availability is non-physiologically low for protein synthesis to occur. To address this issue, Russel-Jones *et al.* (1995) used an amino acid clamp protocol to maintain amino acids in circulation while evaluating the effect of an IGF-I infusion. IGF-I produced an increase in the non-oxidative leucine disposal rate (protein synthesis) with no effect on protein degradation. It was postulated that the actions of IGF-I on protein metabolism may depend on the availability of amino acids. IGF-I may reduce proteolysis during low amino acid availability but causes an increase in protein synthesis with increased amino acid availability. From this, it may be concluded that IGF-I may serve as a link between short term metabolic control (insulin) and longer term regulation of growth and body composition (GH) (Simpson *et al.,* 1998).

The involvement of IGF binding proteins and ALS in metabolic processes involve the sequestering of IGF-I activity a well as, perhaps, functions independent of IGF-I. As mentioned earlier, IGFBPs bind IGF-I. IGFBP-3, ALS and IGF-I form the main ternary

Table 2.1 Summary of *in vivo* effects of IGF levels on carbohydrate, protein and fat metabolism

***in vivo* Treatment**	**Effects**
increased IGF-I ⇒ (injection or infusion)	- decreased GH secretion - decreased insulin response - increased glucose metabolism - increased glucose consumption - increased glucagon secretion - increased sensitivity to insulin - decreased insulin release - decreased thyroid hormone response - decreased plasma triglyceride, β-hydroxybutyrate and free fatty acids - decreased proteolysis - decrease in plasma leucine and α-ketoisocaproate - decrease in overall protein breakdown - increased protein synthesis
decreased IGF-I ⇒ (gene deletion or transgenics)	- increased GH secretion - reduced IR levels and IR/IRS phosphorylation - decreased glucose removal - increased insulin secretion - decreased fat mass - increased leptin levels - increased cholesterol

Abbreviations: abbreviations given in the List of Abbreviations on page xii

complex found in circulation comprising 80-90% of all bound IGF-I. The other binding proteins make up the remaining 10-20% forming binary complexes without ALS. IGF- I complexes inhibit the hypoglycemic potential of free IGF-I as well as the effects on lipid and protein metabolism. Therefore, in formation of IGF-I complexes, IGFBPs and ALS contribute to the metabolic status of the individual. IGFBP-1 was the first binding protein discovered and has been the subject of most studies. Recently, much attention has been paid to IGFBP-1 because of its potential physiological role in metabolism, diabetes and its regulation by insulin. IGFBP-1 levels are inversely related to insulin levels (Cotterill *et al.,* 1989). In normal healthy adults, IGFBP-1 expression follows a diurnal variation with highest levels overnight when insulin secretion is the lowest (Holly *et al.,* 1988). Experiments with rhIGFBP-1 injection have shown that IGFBP-1 induces hyperglycemia; this condition was abolished with co-injection of IGF-I and amniotic IGFBP-1 (Lewitt *et al.,* 1991). IGFBP-1 infusion reduces plasma free IGF-I (Lang *et al.,* 2003) and its hypoglycemic effect due to its association with IGF-I. The administration of rhIGFBP-1 in rats showed a stimulation of insulin release without any effect on glucose levels. In this case, IGFBP-1 was bound to free endogenous IGF-I thus lowering its active concentration (Mortensen *et al.,* 1997). The decrease of IGF-I levels through *IGF-I* gene deletion in mice has also been shown to increase insulin levels (Isaksson *et al.,* 2001). Absence of changes in glucose levels may be partially due to the absence of phosphorylation of rhIGFBP-1 (Frystyc, 2000). This will be discussed further in a later section. Recently, IGFBP-7, a low affinity IGF binding protein, was found to be a high affinity insulin binding protein (Yamanaka *et al.,* 1997).

2.2 Structure and Regulation of Expression of Components of the IGF-I System

2.2.1 IGF-I

The *igf-I* gene is located on human chromosome 12 (Barreca *et al.,* 1989). The gene sequence contains six exons and 5 introns spanning 80 kb of chromosomal DNA (Rotwein *et al.,* 1986). Exons 3 and 4 encode a portion of the N-terminal leader peptide, the complete sequence of the mature protein and a portion of the C terminus (E domain).

Exons 1, 2, 5 or 6 can be alternatively spliced leading to four splice variants or mRNA subtypes containing differences in the 5' and 3' untranslated regions (UTR). Exon 1 or exon 2 can form the N-terminal sequence giving rise to subtype distinction type 1 or type 2, respectively. The C-terminus sequence can be formed by either exon 5 or 6 giving rise to the distinction Ea or Eb, respectively. Therefore, the 4 subtypes are referred to as 1/Ea, 1/Eb, 2/Ea and 2/Eb. IGF-I mRNA expression of all four subtypes is seen in all tissues in early development but the primary subtype is 1/Ea. At postnatal stages of development, the 1/Ea becomes more prominent in expression especially in the GH responsive liver tissue. The IGF-I gene sequence has been highly conserved amongst species and the existence of different mRNA subtypes within the same species may indicate a functional significance (Yakar *et al.,* 2002). Two promoter regions in the IGF-I gene sequence have been identified to date and are termed P1 and P2 (Nolten *et al.,* 1994).

IGF-I is a single chain polypeptide composed of 70 amino acid residues having a MW of 7.6 kDa. The IGF-I sequence is known for a number of different species and is shown to be highly conserved with few exceptions. It has a 70% sequence homology with IGF-II and a 50% sequence homology with pro-insulin. The protein has 4 domains with domain A and B having the most homology with insulin (60 to 70% similarity). There are three intrachain disulfide bridges and the C and D domains have no homology with pro-insulin and form the carboxy terminus of the protein (Figure 2.2) (Daughaday *et al.,* 1989). The ternary structure of the mature IGF-I has not been determined by X-ray crystallography but is thought to have a similar structure to insulin due to the high sequence similarity.

The biological effects of IGF-I can be modulated through a number of regulatory principles: Firstly, the number of IGF-IRs expressed on the cell surfaces of target cells; secondly, interaction with IGFBPs and ALS which extend IGF half-life, protect from IGF degradation, facilitate IGF transport into distinct body compartments and interfere positively or negatively with IGF-IR interaction; and finally the availability of IGF due to the regulation of its own expression. It has been known for some time that during prenatal and postnatal growth and development, serum IGF-I concentration varies from 20 ng/mL

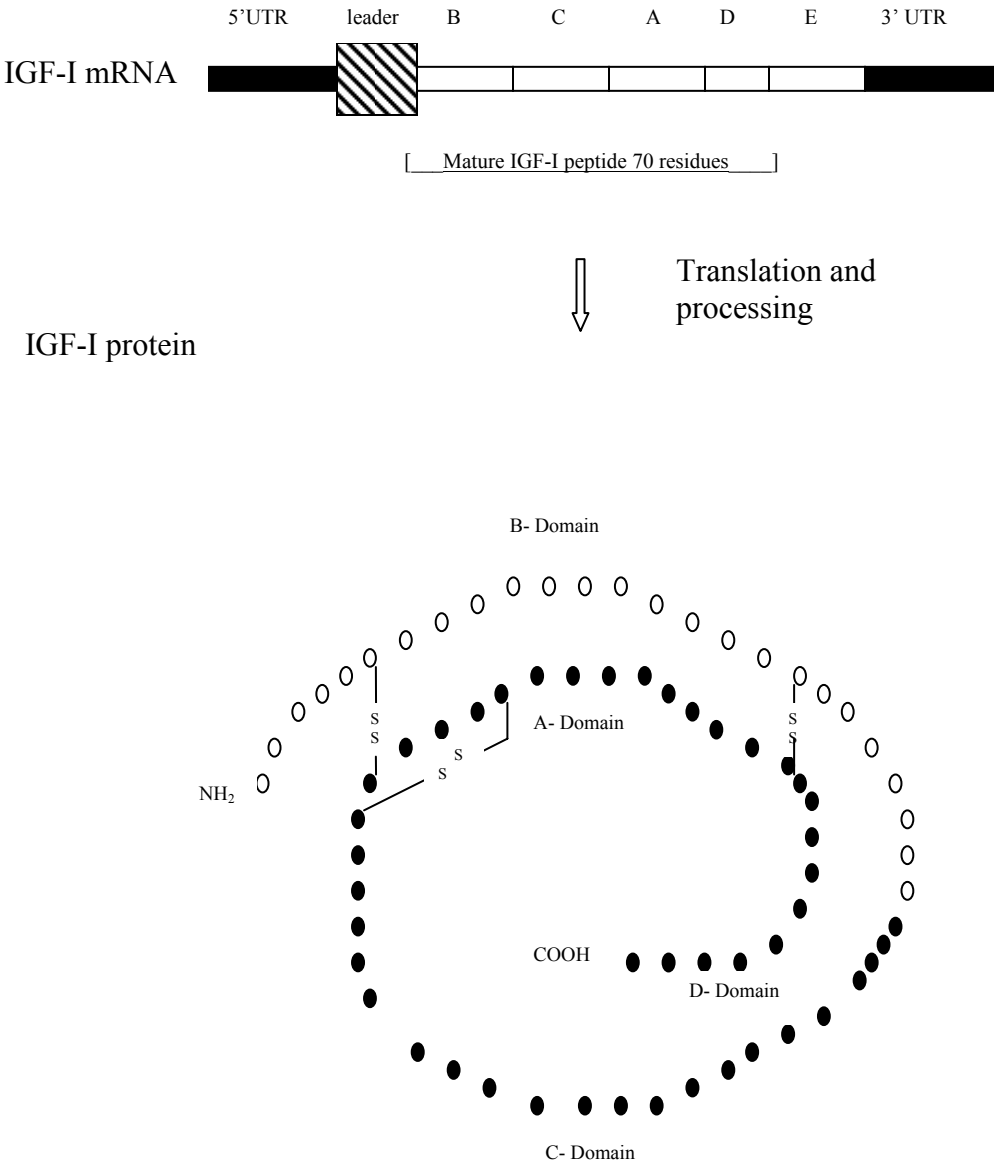


Figure 2.2 Schematic illustration of the IGF-1 mRNA transcript and protein structure. The IGF-I mRNA subtype is not shown for simplification. The mRNA transcript possesses a 5'UTR and leader sequence derived primarily from exons 1 or 2 depending on the mRNA subtype. A 3'UTR is derived from exons 5 or 6 also dependant on the mRNA subtype. Exons 3 and 4 encode B, C, A, D and E domains which are translated into pre-protein. Proteolytic cleavage of the C-terminal E domain results in the formation of a mature protein. The protein contains three disulfide bridges. X-ray crystallography has not been done to determine 3-D structure, but due to sequence similarities structure is thought to resemble that of insulin.

in fetal stages to 200 ng/mL after birth (Bennet *et al.,* 1983, Rotwein 1999). This large increase reflects a strong IGF-I regulation pattern and has lead to extensive research in this area. Several studies have elucidated promoter sequences, transcription factors and a number of hormones and signaling molecules involved in regulation of IGF-I (Table 2.2). Although IGF-I is expressed in many tissues, this section will primarily review regulation of IGF-I expression in hepatic tissue.

Production of IGF-I in liver is relatively insignificant in early development. Postnatal hepatic expression of IGF-I increases to a maximum at birth and then decreases with age. A second and less prominent peak occurs during puberty. There are a number of factors which have been found to regulate the bioactive levels of hepatic derived IGF-I. Insulin has been found to increase liver IGF-I transcription *in vivo* (Pao *et al.,* 1992) and in primary hepatocyte cultures (Pollak *et al.,* 1989). GH also has been found to increase IGF-I mRNA in primary hepatocytes (Norstedt *et al.,* 1987) and in the liver (*in vivo*) (Bichell *et al.,* 1992). Glucagon increases IGF-I mRNA in primary hepatocytes (Kachra *et al.,* 1991) whereas estrogen increases transcription of IGF-I in HepG2 hepatoma cells (Umayahara *et al.,* 1994). The thyroid derived hormone, triiodo-L-thyronine (T_3), increases IGF-I mRNA levels in both primary hepatocyte cultures (Tollet *et al.,* 1990) and in liver (*in vivo*) (Wolf *et al.,* 1989). Hormones can regulate protein levels through regulation of transcription, post-transcriptional processes that govern formation, stability and translation, and post-translational processes such as proteolytic cleavage and glycosylation. It is not yet clear the methods the above mentioned hormones utilize in IGF-I regulation. However, a number of the mentioned hormones will be discussed in detail later in this thesis.

The IGF-I gene contains two promoter sequences, P1 and P2, which are located upstream of exon 1 and exon 2, respectively and do not contain TATA and CAAT elements (Sussenbach *et al.,* 1989). To date, three liver specific transcription factors (TF) have been identified. These three TFs bind to six binding sites located in the P1 promoter and after birth are expressed at much higher levels in the liver than any other tissue. The roles of liver specific TFs, as well as other more ubiquitous TFs, have been studied using transient transfection assays. The first TF, the CAAT- box / enhancer binding protein

Table 2.2 Modulators of IGF-I expression

Molecule	Class	Effect on IGF-I Expression	Reference
Insulin	Hormone	Increases IGF-I mRNA	Pao *et al.,* 1992 Pollak *et al.,* 1989
GH	Hormone	Increases IGF-I mRNA	Norstedt *et al.,* 1987 Bichell *et al.,* 1992
Glucagon	Hormone	Increases IGF-I mRNA	Kachra *et al.,* 1991
Estrogen	Hormone	Increases IGF-I mRNA	Umayahara *et al.,* 1994
T3	Hormone	Increases IGF-I mRNA	Tollet *et al.,* 1990 Wolf *et al.,* 1989
C/EBP (α,B)	Transcription factor	increase promoter activity (liver)	Nolten *et al.,* 1994
HNF-1α	Transcription factor	increase promoter activity (liver)	Nolten *et al.,* 1995
HNF-3β	Transcription factor	increase promoter activity (liver)	Nolten *et al.,* 1996
cAMP	Signaling molecule	increase promoter activity (liver)	De Simone *et al.,* 1992
AP-1	Transcription factor	increase promoter activity (liver	De Simone *et al.,* 1992
ETF	Transcription factor	increase promoter activity	De Simone *et al.,* 1992
glucocorticoid	Hormone	Increases IGF-I mRNA	De Simone *et al.,* 1992

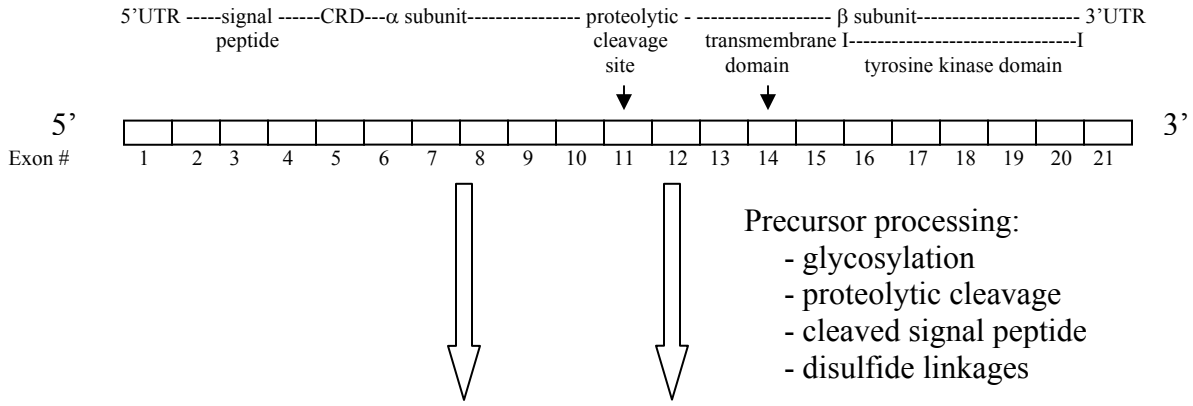
Abbreviations: abbreviations are given in the List of Abbreviations on page xii

(C/EBP), has two binding sites on P1. Two subtypes exist, C/EBPα and C/EBPβ, which bind to either of the two P1 binding sites and increase promoter activity (Nolten *et al.,* 1994). The other two TFs belong to the hepatocyte nuclear factor (HNF) family. HNF-1, and more specifically the HNF-1α isotype, bind to two sites on the P1 promoter (Nolten *et al.,* 1995). The other liver specific HNF is HNF-3 with the isoform HNF-3β being the strongest activator. This TF binds to two binding sites on P1 (Nolten *et al.,* 1996). Presence of these liver-specific TFs may help in explaining why the liver has developed as the source of endocrine IGF-I. Other more ubiquitous TFs exist which may also be active in hepatocytes. One example is those TFs which act downstream of the cAMP signal which interact with the cAMP response element (CRE) within the untranslated region of exon 1 of the IGF-I gene. Others TFs include adaptor protein (AP)-1, E2F and glucocorticoid (De Simone *et al.,* 1992).

2.2.2 IGF-I Receptor

The IGF-IR gene consists of 21 exons arranged in a 100 kb span at the distal end of human chromosome 15 (Abbott *et al.,* 1992). Exons 1-10 contain a 5' UTR, a signal peptide sequence, a cysteine rich domain (CRD) and the remaining sequence of the α subunit. A proteolytic cleavage site exists within exon 11 and exons 12-21 code for the β subunit. In the β subunit portion of the gene exon 14 codes for a transmembrane domain and exons 16-20 code for the tyrosine kinase domain (Cohick *et al.,* 1993) (Figure 2.3). Comparison of the IGF-IR and IR genes show that 12 out of the 21 exons are identical in size. In the other existing exons the differences do not exceed 15 nucleotides. The highest similarity in sequence (84%) is found in the tyrosine kinase domain. Significant homology also exists between the non-cysteine rich portion of the α subunit (64%) and the cysteine rich regions (45%) (Werner 1999). The mRNA transcript of the IGF-IR gene is seen as an 11 kb band on a Northern blot (Lowe *et al.,* 1990). The transcript includes a 1 kb 5' UTR, a 5.1 kb coding region and a 5 kb 3' UTR. The IGF-IR has been identified in virtually every tissue, except the liver, and regulation depends on developmental stage. During early embryonic growth stages transcription levels are high and decrease

IGF-IR mRNA structure



IGF-IR protein structure

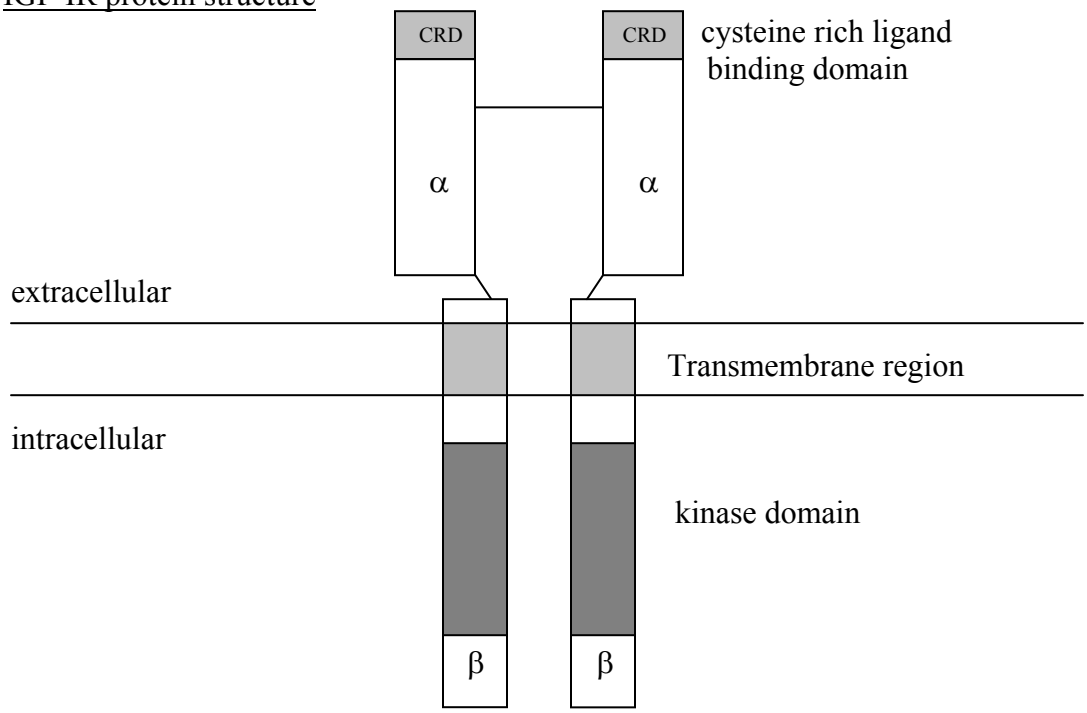


Figure 2.3 IGF-1R mRNA structure, processing and protein structure. IGF-1R functional domains are indicated above corresponding to numbered exons. Upon translation the pre-peptide is processed through glycosylation, proteolytic cleavage and addition of disulfide linkages (——). The α and β subunits are assembled and expressed on the cell membrane with the α subunit exclusively extracellular and the β subunit primarily intracellular but also contains a transmembrane region and a small extracellular region.

Abbreviations: abbreviations are given in the List of Abbreviations on page xii

postnatally with lowest expression observed in adults (Werner *et al.,* 1989). The liver produces the highest amount of IGF-I but produces the least IGF-IR. IGF-I may downregulate expression of its own receptor. High levels of IGF-IR mRNA have also been seen in de-differentiated states. For example, rat granulosa cells transfected with simian virus-40 (SV-40) show marked increases in IGF-IR expression (Werner *et al.,* 1989).

The IGF-IR is translated and processed to synthesize a 180 kDa glycoprotein (Figure 2.3). Precursor processing involves the removal of a polar residue rich signal peptide and core glycosylation. The proreceptor partial processing involves terminal glycosylation, disulfide linkages as well as proteolytic cleavage at a basic tetrapeptide sequence (Arg-Lys-Arg-Arg). This gives rise to the α and β subunits which are synthesized co-linearly from a single precursor molecule composed of 1367 amino acids (Ullrich *et al.,* 1986). Structurally, the α subunit is entirely extracellular. The sequence contains 11 N-linked glycosylation sites as well as a cysteine rich region (24 cysteine residues) which is important for binding to IGF-I. The β subunit spans the membrane with a large cytosolic portion. The extracellular portion contains 5 N-linked glycosylation sites and is 194 residues in length. The transmembrane region consists of 24 hydrophobic residues. The cytoplasmic portion of the β subunit contains a 256 amino acid tyrosine kinase domain with a conserved glycine rich region important for phosphate transfer to specific substrates (Werner 1999).

The regulation of IGF-IR expression occurs mainly at the transcriptional level. The IGF-IR gene contains control elements found in both constitutive and regulated genes. IGF-IR, therefore, is expressed in every cell type but is regulated. The promoter region, like the IGF-I promoter, does not contain the CAAT and TATA motifs and is similar to other promoter sequences which have been termed as 'housekeeping genes' (Werner *et al.,* 1990). In comparison to the IR, the IGF-IR is unique. The IGF-IR promoter contains a single 'initiator' motif that can direct transcription in the absence of the TATA and CAAT *cis*-elements normally required for proper transcription. The IR also lacks the TATA and CAAT elements but contains multiple initiator sites (Araki *et al.,* 1987). The existence of the initiator motif has been found in promoters of genes which are highly regulated during differentiation and development. The promoter

sequence is G and C rich (Cooke *et al.,* 1991) and contains multiple G-C boxes which are potential binding sites for the SP1 and the early growth response (EGR) (including WT-1) families of TFs (Courey *et al.,* 1992, Sukatme *et al.,* 1992). SP-1 binds G-C boxes in the IGF-IR gene promoter through three zinc-finger domains and stimulates IGF-IR expression (Courey *et al.,* 1988). In addition, the 5' flanking region of the IGF-IR gene contains potential binding sites for GC factor (GCF), AP-2 and ETF (Werner *et al.,* 1990, Cooke *et al.,* 1991).

Local and circulating levels of a number of hormones and growth factors exhibit a control on IGF-IR expression. IGF-I itself regulates IGF-IR expression. Increased IGF-I concentrations result in decreased IGF-IR levels. The mechanism by which IGF-I regulates IGF-IR is likely through a translocation or downregulation of the cell surface IGF-IR to an intracellular pool. This downregulation is reversed with decreased IGF-I levels (De Souza *et al.,* 1995). Increased IGF-IR has been associated with growth disorders such as Laron-type dwarfism and caloric deprivation both due to decreased IGF-I levels. The presence of free IGF-I is therefore significantly controlled through the level of IGF-IR expression. Control elements which cause this effect on IGF-IR expression have not been determined. Other growth factors and hormones also have an effect on IGF-IR expression. Stimulatory factors are PDGF and FGF whereas insulin and estrogen are inhibitory factors (Werner 1999) (Table 2.3).

Higher levels of IGF-IR expression have been found in most tumours and cancer cells and have been implicated in malignant transformation. This was demonstrated in NIH3T3 fibroblasts overexpressing functional IGF-IR. Results indicated a ligand dependent, highly transformed phenotype that resulted in tumour formation in nude mice (Kaleco *et al.,* 1990). Truncation or mutation of the IGF-IR gene results in its inability to transform transfectants suggesting that the IGF-IR is a target for multiple oncogenic agents such as the *c-myb* protooncogene (Travali *et al.,* 1991) and the src oncogene which encodes $pp60^{src}$. The effect of transformation of human cells by $pp60^{src}$ results in constitutive tyrosine phosphorylation of the IGF-IR β-subunit. Of the expressed IGF-IR unstimulated tyrosine kinase domains, 10% to 50% are phosphorylated leading to activation of downstream mitogenic pathways (Kozma *et al.,* 1990). Regulation of the IGF-IR gene also occurs through tumour suppressors. Tumour suppressors are mutated in

a large number of human cancers where IGF-IR is overexpressed. The absence of functional tumour suppressor therefore transcriptionally derepresses IGF-IR expression leading to its over-expression and downstream mitogenic responses in these cells. Examples of tumour suppressors which exhibit this activity are WT1 (Werner *et al.,* 1993 Werner *et al.,* 1994) and mutant p53 (Oren 1992) (Table 2.3).

Table 2.3 Modulators of IGF-IR expression

Molecule	Class	Effect on IGF-IR Expression	Reference
SP-1	Transcription factor	G-C box binding increases expression	Courey *et al.,* 1988 Beitner-Johnson *et al.,* 1995
WT-1	Transcription factor	tumour suppressor G-C box binding decrease expression	Sukatme *et al.,* 1992 Werner *et al.,* 1993
GCF, AP-2, ETF	Transcription factors	Not yet determined	Werner *et al.,* 1990 Cooke *et al.,* 1991
IGF-I	Hormone	Negatively regulates unknown mech.	De Souza *et al.,* 1995
PDGF, FGF and estrogens	Hormones	Increase expression	Werner 1999
Insulin, estrogen	Hormones	Increase expression	Werner 1999
c-myb proto-oncogene	oncogene	Increase expression	Travali *et al.,* 1991
src oncogene	$pp60^{src}$ protein	IGF-IR phosphorylation mitogenic pathway activation	Kozma *et al.,* 1990
p53	tumour suppressor transcription factor	Decreased transcription	Oren 1992

Abbreviations: abbreviations are given in the List of Abbreviations on page xii

2.2.3 IGF Binding Proteins

2.2.3.1 IGFBP-1

IGFBP-1 is encoded by a single copy gene on human chromosome 7p14-p12 (Figure 2.4) (Ekstrand *et al.,* 1990). The gene spans 5.2 kb and contains 4 exons. The larger size of exon 1 is a characteristic of all IGFBPs. The IGFBP-1 gene sequence is found contiguous with IGFBP-3 in a tail to tail conformation. A 24 bp direct repeat exists between intron 3 and exon 4 and a 3' acceptor splice site has been identified in the same region, 93 bp upstream of exon 4 (Allander *et al.,* 1993). The promoter region of the IGFBP-1 gene extends -1205 bp upstream of the transcription initiation site which exists nt -28 bp upstream of the translation initiation site or the ATG codon. The transcribed IGFBP-1 mRNA product consists of a 56-164 bp 5'UTR, 564-612 bp 3'UTR, a coding region of 798 bp and a consensus poly A signal (AATAAA) followed by a poly (A) tail (Figure 2.4). A single mRNA transcript for IGFBP-1 with a size of 1.5 kb has been determined by Northern analysis (Cubbage *et al.,* 1989). IGFBP-1 cDNA has been isolated from human deciduas, liver, placenta and HepG2 hepatocarcinoma cells (Guck *et al.,* 1999).

The isolated IGFBP-1 cDNA encodes a 259 amino acid precursor, with a 25 residue signal peptide and a 234 residue mature polypeptide. The apparent molecular mass of IGFBP-1 is 32-34 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Rechler 1993). The amino acid sequence contains 18 cysteine residues mainly localized in clusters within the C and N-terminus which form disulfide linkages in the protein ternary structure (Brinkman *et al.,* 1991a and b). There are no sites for N-glycosylation, but the protein sequence does contain a RGD (Arg-Gly-Asp) motif at the C-terminus; a consensus sequence for the binding of proteins to cell surface integrin molecules. Similar RGD sequences have been identified and studied in fibronectin and matrix adhesion molecules (Ruoslahti *et al.,* 1987, 1991, Jones *et al.,* 1993). Also within the C-terminal sequence are PEST (Pro-Glu-Ser-Thr) motifs which have been found to be common in rapidly degraded proteins

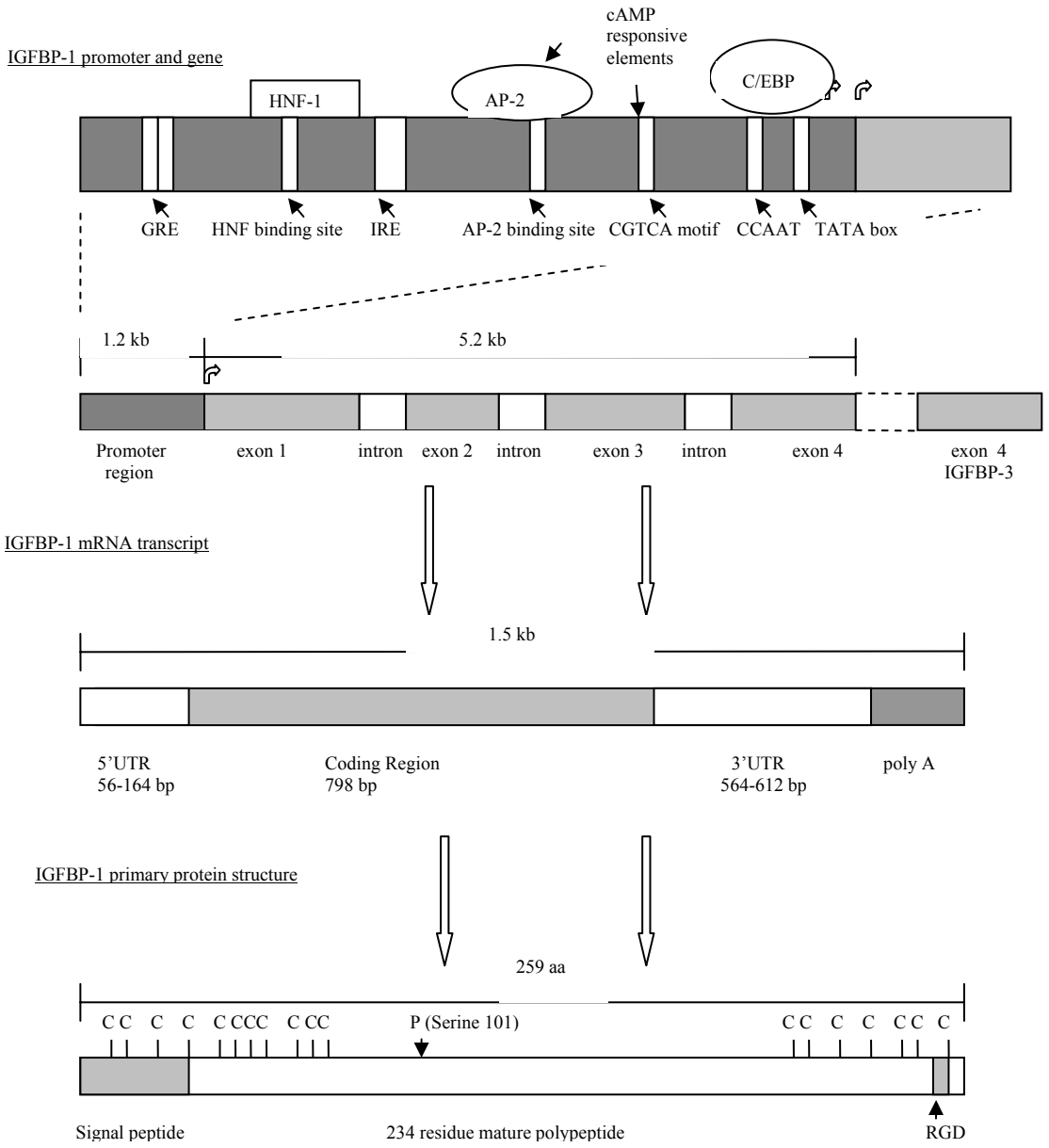


Figure 2.4 Schematic of IGFBP-1 promoter, gene, mRNA and primary protein structure. The promoter region is shown with TF binding sites indicated as well as important regulatory elements mentioned in the text. The gene consists of 1.2 kb promoter and 5.2 kb region containing 4 exons and 3 introns. 1.5 kb mRNA transcript consists of a 5'UTR, coding region, 3'UTR and Poly A tail. The 259 amino acid (aa) primary protein structure as shown above has cysteine rich domains in the C- and N- terminal regions. Also shown are the relative locations of Ser 101, the signal peptide and the RGD domain.

Abbreviations: C, cysteine; P, Phosphorylation; aa, amino acids; for remaining abbreviations see List of Abbreviations pg. xii

(Julkunen *et al.,* 1988). Binding of IGF-I to IGFBP-1 in circulation is somewhat dependent on the degree of IGFBP-1 phosphorylation. Non-phosphorylated IGFBP-1 has a lower affinity for IGF-I as compared to phosphorylated IGFBP-1 (Jones *et al.,* 1991). The non-phosphorylated IGFBP-1 might have a stimulatory role rather than an IGF inhibitory role (Wetterau *et al.,* 1999). In humans, residue Ser 101 is phosphorylated and is an important site for the IGFBP-1 affinity to IGF-I. This residue does not have any role in rat and mouse (Lee *et al.,* 1994).

In human serum, IGFBP-1 shows dynamic regulation. *In vivo* studies have indicated that developmental and metabolic statuses regulate IGFBP-1 levels in humans and rats. During development, IGFBP-1 levels are elevated in fetal serum but decrease by a factor of ten at term and then gradually decrease following birth to a steady state level at puberty (Drop *et al.,* 1984). The regulatory effect of metabolic status on IGFBP-1 expression is mediated through insulin levels. IGFBP-1 expression is suppressed by increased insulin levels (Suikkari *et al.,* 1989a). Metabolic conditions which cause decreased insulin levels such as diabetes, fasting and prolonged exercise cause opposite effects on IGFBP-1 levels (Hilding *et al.,* 1991, Brismar *et al.,* 1988, Busby *et al.,* 1988, Suikkari *et al.,* 1989b). Glucose intake may have a positive effect on IGFBP-1 expression, but the effect is probably independent of insulin (Snyder *et al.,* 1990). In adults with GH deficiency, IGFBP-1 levels are higher suggesting that GH may have a suppressive effect (Busby *et al.,* 1988). Along with metabolic and developmental regulation of IGFBP-1 levels, it has also been shown that IGFBP-1 expression occurs in the uterine decidua where IGFBP-1 levels can increase five-fold during pregnancy (Rutanen *et al.,* 1985). IGFBP-1 levels in human amniotic fluid can increase to 1000-fold than that of serum concentration at midterm pregnancy (Guck *et al.,* 1999).

Along with *in vivo* studies, *in vitro* regulation of IGFBP-1 expression has also been shown with the use of a number of cells. IGFBP-1 expression has been shown in liver-derived hepatoma cell lines (H4IIE, HepG2) as well as in breast cancer and endometrial adenocarcinoma cells. The H4IIE rat hepatoma cells have been utilized for a number of studies with different potential effector molecules. It has been found that physiological amounts of dexamethasone increase H4IIE IGFBP-1 mRNA abundance 10-fold (Orlowski *et al.,* 1990). Insulin and cAMP inhibit H4IIE cell IGFBP-1 protein

expression (Unterman *et al.,* 1991). GH has no effect in H4IIE cells (Orlowski *et al.,* 1991) although some studies have shown a transcriptional repression of IGFBP-1 expression by GH in rat liver (Seneviratne *et al.,* 1990). Similar patterns of IGFBP-1 regulation by the above mentioned metabolic modulators have been shown in liver tissue explants, rat hepatocyte culture and HepG2 cells (Guck 1999).

Many IGFBP-1 gene promoter *cis* regulatory regions have been identified for transcription factors in hepatocytes (Table 2.4, Figure 2.4). The IGFBP-1 promoter region is located within -1205 bps upstream of the transcription initiation site. The promoter contains a TATA box at nt -28 and a CCAAT sequence at nt -72 to which the C/EBP TF may bind and affect expression. Analysis of the promoter region through a progressive 5' deletion indicated a basal promoter activity dependent on a *cis*-element which binds HNF-1. This element is highly conserved in several species and when abolished or mutated basal activity is decreased (Suwanichkul *et al.,* 1990).

There have also been studies indicating the involvement of a number of TFs which mediate hormonal regulation of the human IGFBP-1 promoter. It has been proposed that IGFBP-1 has a glucoregulatory role (Lewitt *et al.,* 1991). The similarity of IGFBP-1 hormonal regulation to that of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis, strengthens this proposition (O'Brien *et al.,* 1990a and b). Within the human IGFBP-1 promoter, between nt -263 and 254, a CGTCA motif exists which is known to mediate the effect of cAMP on a number of different promoters (Montminy *et al.,* 1990). Mutation of this motif results in a 51% decrease in cAMP stimulated promoter activity (Suwanichkul *et al.,* 1993a). An AP-2 binding site has been identified in the rat IGFBP-1 gene promoter. It has been indicated that cAMP may effect expression through the AP-2 binding *cis*-element and TF (Roesler *et al.,* 1988, Suh *et al.,* 1994). The positive regulation of the IGFBP-1 gene to dexamethasone is attributed to the presence of two glucocorticoid response elements (GREs) located in the first 200 bp of human IGFBP-1 promoter sequence. The GRE sequences have week affinity for the glucocorticoid receptor and the loss of either of the two elements results in complete loss of activation (Suwanichkul *et al.,* 1994). The IGFBP-1 promoter contains an insulin response element (IRE) located as a palindromic sequence in both rat and human (Suh *et*

Table 2.4 Modulators of IGFBP-1 expression

Molecule	Class	Effect on IGFBP-1 Expression	Reference
Insulin	Hormone	Decrease expression	Suikkari *et al.,* 1989
Glucose	Carbohydrate	Undecided	Snyder *et al.,* 1990
GH	Hormone	Decrease expression	Busby *et al.,* 1988
Dexamethasone	Hormone	Increase mRNA	Orlowski *et al.,* 1990
cAMP	Signaling molecule	Inhibits expression	Unterman *et al.,* 1991
C/EBP	Transcription factor	Undecided	Suwanichkul *et al.,* 1990
HNF-1	Transcription factor	Maintains level of basal transcription	Suwanichkul *et al.,* 1994
AP-2	Transcription factor	cAMP mediated regulation ?	

Abbreviations: abbreviations given in the List of Abbreviations on page xii

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al., 1994). The IRE sequence was first discovered in the PEPCK gene promoter region and was comprised of a 10 bp sequence located between nt -416 and -407 (O'Brien *et al.,* 1990a). Mutation of one of the IGFBP-1 IRE palindromic sequences slightly decreases insulin mediated effects on transcription, but abolishing the sequence entirely leads to loss of all inhibition of promoter activity by insulin (Suwanichkul *et al.,* 1993b). The commonality of the IRE between IGFBP-1 and PEPCK may explain the dynamic regulation by insulin seen for both of these proteins.

2.2.3.2 IGFBP-3

The human IGFBP-3 gene exists on chromosome 7 separated 20 kb from the IGFBP-1 gene in a tail-to-tail conformation (Ehrenborg *et al.,* 1992). The gene spans 8.9 kb and contains four exons. Unlike the other IGFBPs, IGFBP-3 contains most of the 3' untranslated region and polyadenylation signal on an additional fifth exon. The IGFBP-3 promoter has been identified and characterized (Cubbage *et al.,* 1990). The promoter contains proximal GC boxes as well as a TATA box. Northern analysis has identified a single mRNA sequence of 2.5 kb (Shimasaki *et al.,* 1989) which is conserved in a number of species.

The IGFBP-3 cDNA predicts a 291 amino acid precursor, with a 27 residue signal peptide and a 264 residue mature polypeptide (Wood *et al.,* 1988). The peptide sequence contains 18 cysteine residues and three potential N-glycosylation sites. On non-reducing SDS-PAGE gels IGFBP-3 has shown migration in doublets of 40-50 kDa which reflect different levels of N-glycosylation (Rechler *et al.,* 1993). It has also been found in Chinese hamster ovary (CHO) cells that the mature IGFBP-3 protein contains phosphorylation on serine 111 and 113 (Hoeck *et al.,* 1994). IGFBP-3 cDNAs have been studied in rat, bovine, porcine and human models with similar characteristics.

During embryonic development IGFBP-3 expression is detectable as early as day 18 in the developing mouse liver and vertebrae (Schuller *et al.,* 1993). In the rat embryo, expression is identified as early as day 14 in the urogenital system and in muscle tissue. By day 21, IGFBP-3 mRNA is detectable in liver, kidney, muscle and the developing cerebral cortex. Endothelial cells, such as those found in rat liver, placenta, white adipose tissue (WAT) and human testis, are particularly abundant in IGFBP-3 expression (Chin *et al.,* 1994). Human *in vivo* study of IGFBP-3 indicates birth levels to be one third of the levels seen in the adult (Bang *et al.,* 1994). In rodents, birth levels are nearly undetectable (Donovan *et al.,* 1989). Post-natally, IGFBP-3 is the most abundant IGFBP in circulation comprising 75-85% of all circulating IGFBPs (Baxter *et al.,* 1986, Baxter *et al.,* 1989). The levels of IGFBP-3 in serum increase to a maximum level during puberty. Marked decreases in IGFBP-1 levels occur during birth, GH insufficiency (Baxter *et al.,* 1986), diabetes (Zapf *et al.,* 1990) and undernutrition (Thissen *et al.,* 1994). IGFBP-3 mRNA

abundance after birth or hypophysectomy show little change in the liver and kidney while exhibiting significant changes in serum IGFBP-3 levels. Regulation of IGFBP-3 synthesis, therefore, appears to occur mainly through translational and/or postranslational mechanisms (Chin *et al.,* 1994, Albiston *et al.,* 1992). *In vitro* studies indicate that Kuppfer and sinusoidal endothelial cells express IGFBP-3 exclusively. In these cells, dexamethasone treatment decreases IGFBP-3 mRNA while insulin increases it (Villafuerte *et al.,* 1995). *In vivo* study suggests IGF-I to be a direct regulator of IGFBP-3 mRNA expression due to its ability to increase serum IGFBP-3 levels in GH deficient rodent models (Zapf *et al.,* 1989). This activity of IGF-I is not seen in humans. However, *in vitro,* within human Kuppfer and sinusoidal cells, IGF-I increases IGFBP-3 half life. This probably explains the *in vivo* effect mentioned above (Villafuerte *et al.,* 1996).

The IGFBP-3 promoter contains a TATA box and a GC box within its sequence. The GC box contains binding sites for Sp1 and AP-2 (Albiston *et al.,* 1995). An IRE has also been identified, but the sequence of this IRE is non-homologous to the IRE found in IGFBP-1. The sequence recognizes a 90 kDa nuclear protein which is upregulated with insulin signal in parenchymal cells and diabetic liver (Villafuerte *et al.,* 1997). The tumour suppressor, p53, was also identified as an IGFBP-3 inducer in EB1 colon carcinoma cells. Two elements in intron 1 and 2 are capable of binding p53 and cause induction through a heterologous promoter (Buckbinder *et al.,* 1995).

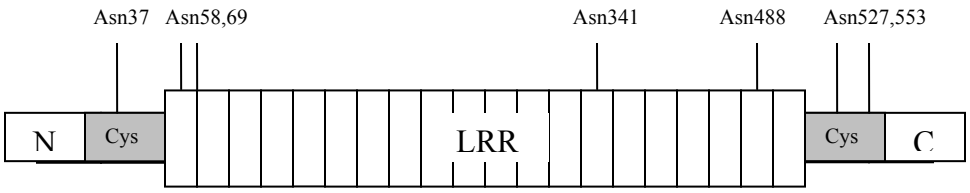
2.2.4 Acid Labile Subunit

The ALS gene exists as a single copy gene which maps to the short arm of chromosome 16 p13-3 (Suwanichkul *et al.,* 2000). In mice, the ALS gene spans 3.3 kb on chromosome 17 and is composed of two exons. Exon 1 encodes 5 amino acids of the signal peptide and exon 2 encodes the entire precursor protein and the remaining 27 amino acids of the signal peptide. There is no TATA box or other initiation elements in the ALS gene, but transcription begins in a region 140 bp upstream from the ATG codon. ALS promoter activity has been identified between nt -2001 and -49 (Boisclair *et al.,* 1996). This chromosomal structure is conserved across species (Boisclair *et al.,* 2001). Transcription of the ALS gene results in a 2.2 kb mRNA transcript. Sequence identity of

mature ALS is 93% between mouse and rat, 79% between rat and human and 73% between mouse and human.

Human cDNA for ALS encodes an ORF of 605 amino acids containing a 27 amino acid signal peptide and a 578 amino acid mature protein (Leong *et al.,* 1992). The primary protein structure contains 18-20 leucine rich domains (LRD) each comprised of 24 amino acids of which 6 are typically leucine residues (Figure 2.5). The leucine rich repeat (LRR) region accounts for 75% of the mature ALS protein. These repeated domains are thought to be important in protein-protein interactions and characterize the LRR superfamily of proteins, of which ALS is a part. Although the crystal structure of ALS has not yet been determined, comparative analysis based on molecular modeling of LRR structures in other LRD containing proteins has been done to determine the ALS tertiary structure. The repeated LRDs give a curvature to the protein conformation. This has been shown for the structure of another leucine-rich repeat family member, the porcine ribonuclease inhibitor (RI). RI is the only LRR superfamily member of which a crystal structure has been solved and it contains only 15 LRR compared to the 18 LRRs found within ALS structure. The RI protein conformation resembles a horseshoe with each repeat containing a β-sheet and a α-helix alternating when a number of repeats are linked. This characteristic secondary structure gives the RI and other LRR proteins a facedness. The internal face is lined with a parallel β-sheet lying perpendicular to the plane of the molecule and the external face is made up of α-helices and loops. The extra LRRs allow ALS to complete a circular/donut shaped structure, akin to the joining of the two ends of the RI horseshoe structure with 3 LRDs (Figure 2.5). This structure is strongly supported through visualization of ALS molecules by electron microscopy (Janosi *et al.,* 1999b). The primary structure also contains 13 cysteine residues and 7 potential N-linked glycosylation sites (Leong *et al.,* 1992). One site for N-linked glycosylation is located in the middle of the ALS sequence and three are located at each terminal end. On SDS- PAGE, ALS migrates as a 84-86 kDa glycoprotein. After enzymatic removal of N-linked sugars, ALS migrates as a 63-66 kDa protein indicating approximately 20 kDa of N-linked sugar content (Baxter *et al.,* 1994). Studies done on ALS N-linked glycosylation have shown that in the proposed donut conformation the six terminal N-linked sugars are

ALS Domain Structure:



ALS Ternary Structure:

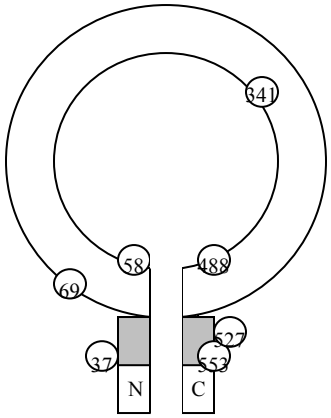


Figure 2.5 Schematic illustration of ALS domain structure and tertiary structure. The domain structure as shown exhibits the central LRR region flanked by cysteine rich regions and N-terminal and C-terminal regions. Also indicated are Asn residues important for the formation of N-linked glycosylations. These glycosylations are indicated on the diagram of ternary structure (see numbered circles). The determined ternary structure resembles a donut which is due to the conformation of LRRs. Structure is inferred from similar structures in other LRR containing proteins. Tertiary structure adapted from Janosi *et al.,* 1999.

Abbreviations are defined in List of Abbreviations pg. xii

clustered and may provide a strong negative charge due to the presence of negatively charged sialic acids (Janosi *et al.,* 1999a). The importance of this charged group will be discussed in section 2.3.

ALS is almost exclusively found in serum. It is first detectable in humans during the late stages of fetal life and then increases 5-fold during puberty and remains constant in adulthood (Baxter 1990a). Subsequently, IGF exists only in the 50 kDa binary complex before birth and is found in the ternary complexes, with ALS, after birth. Postnatally, ALS mRNA is only detectable by Northern analysis in liver parenchymal cells, the principle site of ALS synthesis. The proximal tubule of the kidney, the lung, the thymus and the mammary gland also express ALS but the amounts are negligible (Chin *et al.,* 1994, Dai *et al.,* 1994b). Immunoreactive ALS is present in cerebrospinal fluid, amniotic fluid, milk and lymph, and at low concentrations in peritoneal, synovial, ovarian and blister fluid (Baxter 1990, Xu *et al.,* 1995, Cwyfan-Hughes *et al.,* 1997, Khosravi *et al.,* 1997, Labarta *et al.,* 1997). Typical concentrations of ALS in human and rat serum, respectively, are 230 and 570 nM (Baxter 1990a, Khosravi *et al.,* 1997). Serum is the main source of extravascular ALS but local production may also occur. Reduction of serum ALS levels has been shown due to a variety of conditions in rats and humans. Conditions include: fasting, under nutrition and catabolic diseases such as diabetes, burn injury and cirrhosis (Boisclaire *et al.,* 2001).

GH is the most potent *in vivo* hormonal inducer of ALS expression in circulation. The increased ALS expression seen after birth is concomitant with the increases in GH levels detected during the same developmental period (Boisclaire *et al.,* 2001). The pituitary gland is the principle source of GH in animals. With removal of the pituitary gland (i.e. hypophysectomy) liver ALS mRNA levels decrease dramatically (Dai *et al.,* 1997). Circulating levels of ALS decrease in adult humans with GH deficiency, GH receptor defects and chronic IGF-I infusion. High IGF-I levels cause a feedback inhibition on GH secretion from the pituitary gland therefore decreasing ALS expression (Labarta *et al.,* 1997). Decreases in ALS expression have also been linked to GH resistance in the liver. Studies have shown that the development of GH resistance is accompanied by an increase in the negative actions of cAMP and inflammatory cytokine interleukin (IL)-1β on ALS expression (Delhanty 1998a and b). In primary hepatocytes,

insulin induces ALS secretion without any change in ALS mRNA levels indicating a post-translational mechanism of regulation (Dai *et al.,* 1994a). Decreased ALS levels in diabetes, undernutrition and fasting may be due to decreased levels of insulin in these metabolic conditions. Decreased ALS expression also occurs through direct regulation at the transcriptional level by dexamethasone, cAMP, and epidermal growth factor (EGF) (Werner 1999) (Table 2.5). This has been shown in primary hepatocyte culture and may explain the decreased levels of ALS expression in conditions such as thermal damage and liver damage which are associated with increased internal glucocorticoid and cAMP. *In vitro* mRNA and protein expression of ALS are unsuccessful in a number of liver cells such as H4IIE, H35, HTC, 5123TC, FAO, BRL3A and human HepG2 cells (Guck *et al.,* 1999). The reason for lack of ALS gene expression is thought to be due to chromatin modifications such as methylation of CpG islands (Antequera *et al.,* 1990).

Table 2.5 Modulators of ALS expression

Molecule	Class	Effect on ALS Expression	Reference
GH	Hormone	Increase expression	Boisclaire *et al.,* 2001
cAMP	Signaling molecule	Decrease expression	Delhanty 1998
IL-1β	Hormone	Decrease expression	Delhanty 1998
Insulin	Hormone	Increase expression	Dai *et al.,* 1994a
Dexamethasone	Hormone	Decrease expression	Werner 1999
EGF	Hormone	Decrease expression	

Abbreviations: abbreviations are given in the List of Abbreviations on page xii

2.3 IGF Binding Protein Complex Formation and the Regulation of IGF-I Biological Activity

2.3.1. Introduction

Although IGF regulation is directly related to the transcriptional and translational regulation of IGF system components, the regulation of IGF activity is ultimately due to the ability of the IGFBPs and ALS to bind IGF and form binary and ternary complexes. The formation of IGF binding protein complexes stabilizes IGF in circulation and potentiates or inhibits its ability to act on target tissues. Conserved structural characteristics of IGFBPs indicate the importance of these conserved domains in IGF binding. Other factors related to IGF binding by the IGFBPs are ionic strength, pH glycosylation, phosphorylation and IGFBP proteolysis. Knowledge of the chemistry of IGF and IGFBP association and the subsequent effects on IGF bioactivity is limited, but the available information will be reviewed with emphasis on the IGFBP-1 binary complex and the IGFBP-3 and ALS containing ternary complex. The view that ALS and IGFBPs simply bind and inhibit IGF-I and II activity is very simplistic. Consideration must be taken for the nature or degree of the modulation depending on the IGFBP present, upstream modifications to the IGFBPs themselves, IGFBP proteolysis and IGF independent activities which alter the IGF bioactivity.

Along with IGFBPs, which will be reviewed later in this section, IGF-I has also been studied in relation to IGFBP complex formation. Mutational analysis of IGFBP and IGF interaction has been done with specific mutations in the N-terminal regions of IGF. Substitution of Glu^3 with Arg greatly reduces IGF-I binding to IGFBPs and appears to be particularly important for IGFBP-3 association (Baxter *et al.,* 1992). Insulin binds IGFBP-3 as well, but with 1000-fold less affinity when compared to IGFs (Helding *et al.,* 1994). This characteristic of insulin resulted in the "B chain" mutant IGF-I derivative which substitutes the first 16 insulin residues for the first 16 residues of IGF-I and resulted in highly decreased IGFBP binding affinity (Clemmons *et al.,* 1992). Other IGFs with decreased affinities for IGFBPs include [$Gln^3Ala^4Tyr^{15}Leu^{16}$] IGF-I, also known as

QAYL IGF-I, N-terminally extended forms such as "long" [Arg^3] IGF-I and Des(1-3) IGF-I (Francis *et al.,* 1994, Forbes *et al.,* 1988).

2.3.2 The IGFBP-1 Binary IGF Binding Protein Complex

The estimated combined molecular weight of the IGFBP-1:IGF-I complex is approximately 35 kDa and this complex comprises a small percentage (< 5 %) of all bound IGF in serum. IGFBP-1 expression is highly regulated by insulin and as such, changes in nutrition, fasting-feeding, and diabetes strongly modulate IGFBP-1 levels and the role it plays in IGF regulation. For example, the IGFBP-1 level varies from 10 ng/mL when fed to 100 ng/mL while fasting (Katz *et al.,* 1995). The affinity of IGFBP-1 for IGF-I is slightly higher than for IGF-II and it is partially dependent on phosphorylation. These characteristics are unique to IGFBP-1 as the other high affinity IGFBPs (-2 to -6) have higher affinities for IGF-II than IGF-I and phosphorylation does not have any functional significance. Human plasma derived IGFBP-1 has an affinity for IGF-I and IGF-II of K_a = 2.3 x 10^{10} L/mol and 3.6 x 10^9 L/mol, respectively. These values are derived for the phosphorylated form of IGFBP-1. When dephosphorylated The K_a for IGF-I decreases 10-fold to 2.5 x 10^9 L/mol (Westwood *et al.,* 1997).

There have been no X-ray crystallographic or nuclear magnetic resonance (NMR) structures determined for any IGFBP complex. Any inferences to IGFBP structural domains have been done based on natural variants (i.e. proteolyzed forms), mutagenesis and expression studies (Martin *et al.,* 1999). A naturally occurring N-terminal 21 kDa IGFBP-1 fragment has been isolated from human placenta and was found to have IGF-I binding activity (Huhtala *et al.,* 1986). Separate studies indicated a similar activity with 12 kDa C-terminal IGFBP fragments isolated from human milk which showed IGF-II binding activity (Ho *et al.,* 1997). Confirmation of the importance of these domains in IGFBP-1:IGF-I binding activity was assessed in mutagenesis studies on IGFBP-1. Deletion of 60 N-terminal residues or of 20 C-terminal residues of IGFBP-1 sequence both abolished IGF binding indicating the requirement for both the N- and C-terminal, cysteine-rich domains for IGF binding activity (Figure 2.4) (Brinkman *et al.,* 1991a, 1991b).

IGFBP-1 phosphorylation plays an important role in IGFBP-1 affinity for IGF-I. The human phosphorylated forms of IGFBP-1 have a 10-fold higher affinity for IGF-I than the non-phosphorylated forms (Jones *et al.,* 1991). Also in humans, residue Ser 101 of IGFBP-1 has been shown to be an important phosphorylation site required to maintain affinity for IGF-I. These properties vary amongst IGFBP-1 from different species. For example, the phospho and non-phosphoforms of IGFBP-1 on Ser 101 residues from rat hepatoma cells show no differences in IGF binding activity (Peterkofsky *et al.,* 1998). Similarly, Ser 101 of IGFBP-1 from mice does not have a role (Lee *et al.,* 1994). The phosphorylated IGFBP-1 protein does, however, predominate in circulation. It may be possible that different forms may have different biological activities. In endometrial stromal cells, phosphorylated and non-phosphorylated forms of IGFBP-1 showed equal inhibition of IGF stimulated DNA synthesis through the sequestering of free IGF-I, but progestin-stimulated DNA synthesis was significantly more sensitive to inhibition by the phophorylated form of IGFBP-1 (Frost *et al.,* 1993).

Interestingly, IGFBP-1 has also been shown to potentiate the effect of IGF through enhancement of cellular DNA synthesis in humans, mice and chick embryo fibroblasts (Elgin *et al.,* 1987, Koistinin *et al.,* 1990). Changes in IGF-I affinity for IGFBP-1 are associated with potentiation of IGF effects. This change in affinity may occur through the dephosphorylation of IGFBP-1 (Jones *et al.,* 1991). Thus cellular or extracellular derived dephosphorylated IGFBP-1 may lead to a potentiation of IGF-I activity while the phosphorylated form may lead to an inhibition of IGF-I activity.

Much research has been done regarding the acute modulation of endocrine IGF action by IGFBP-1. In a serum sample, “free IGF-I” comprises approximately 0.5% of total IGF (Frystyk *et al.,* 1994). “Free IGF-I” has a significant hypoglycemic activity. This was first assessed following a bolus injection of human IGFBP-1 into rats and a subsequent transient hyperglycemia (Lewitt *et al.,* 1991). Transgenic rats expressing IGFBP-1 show development of hyperglycemia during fasting (Rajkumar *et al.,* 1995). Injection of human recombinant IGFBP-1 into human subjects causes pulses of insulin secretion, indicative of transient hyperglycemia (Mortensen *et al.,* 1997). Therefore IGFBP-1:IGF-I complex formation appears to block the hypoglycemic activity of free IGF-I. Endogenous IGFBP-1 expression decreases after glucose ingestion and rises in

response to hypoglycemia. Thus, IGFBP-1 counter-regulates the unbound, free IGF-I acutely and inhibits further hypoglycemic activity. It has been shown that serum IGFBP-1 and free IGF-I have an inverse relationship as shown in normal healthy subjects with a bolus of oral glucose (Frystyk *et al.,* 1997). Uptake of glucose in extrahepatic tissues is also affected by IGFBP-1. *In vivo* rat studies have shown that the bolus co-administration of IGF-I and IGFBP-1 slows the disappearance of IGF-I 5-fold but blocks the normal IGF-I induced uptake of glucose by heart and muscle tissues (Lewitt *et al.,* 1993).

The mitogenic effect of IGF-I is also inhibited by IGFBP-1 association. This was first shown in fibroblasts, *in vitro,* with the use of a pure IGFBP-1 preparation from amniotic fluid (Chochinov *et al.,* 1997). Since that time many cell type models have shown similar results in IGFBP-1 inhibition of the IGF-I induced mitogenic effects (Martin *et al.,* 1999). Of interest is that the cell proliferative effects of estradiol are also inhibited by IGFBP-1 in MCF-7 cells that do not secrete IGF-I or II (Figueroa *et al.,* 1993). This may indicate a possible intrinsic bioactivity of IGFBP-1 independent of IGF activities.

IGF-independent activity of IGFBP-1 has been partially explained through IGFBP-1 cDNA analysis revealing the presence of a C-terminal RGD domain. RGD domains have been identified as integrin recognition sequences (Shimasaki *et al.,* 1991). It has been shown that the addition of a synthetic RGD containing peptide blocks binding of IGFBP-1 to cell surfaces (Clemmons 1989). Experiments in Chinese hamster ovary (CHO) cells investigating the binding effects of IGFBP-1 to the $\alpha 5\beta 1$ integrin (fibronectin receptor) have shown a functional significance of IGFBP-1 binding. CHO cell transfection or addition of exogenous human IGFBP-1 both resulted in stimulation of cell migration in monolayer binding assays. Affinity chromatography of ^{125}I- labeled CHO cell surface receptor proteins identified the $\alpha 5\beta 1$ integrin as the only surface receptor protein to bind IGFBP-1 in an RGD dependent manner. Mutation of the RGD sequence to Trp-Gly-Asp (WGD) in IGFBP-1 prevented $\alpha 5\beta 1$ integrin binding and the cell migration effect seen with normal IGFBP-1 (Jones *et al.,* 1993).

IGFBP-1 may also be involved in the transport of IGF-I through the capillary barrier. Evidence suggests that this process may involve an insulin dependent mechanism as insulin differentially alters transcapillary movement of intravascular IGFBP-1 in rat

heart. In rat heart perfusion studies, IGFBP-1 showed an insulin dose dependent increase in movement from the vascular space to heart tissues (Bar *et al.,* 1990). Such insulin-facilitated changes may potentiate nutrient-dependent transport of IGF-I to peripheral tissues.

The regulation of IGFBP-1:IGF-I complex formation is also regulated by the proteolytic activity of proteases specific for the cleavage of IGFBP-1. Although rare in comparison to other IGFBPs, IGFBP-1 fragments have been found in human placenta, milk, human decidua and amniotic fluid. *In vitro* analysis has indicated Cathespin D, Stromelysin-3 and MMP-2, -3, -7 and -11 as proteases able to cleave IGFBP-1 (Binoux *et al.,* 1999). Most IGFBP fragments have reduced affinities for IGF-I when compared to intact protein, with the exception of a 21 kDa carboxy terminal IGFBP-1 fragment which shows similar affinity to IGF-I (Huhtala *et al.,* 1986). Interest has been shown for this protein fragment due to the ability of IGFBP-1 to potentiate IGF-I activity and the possibility that the C-terminal RGD sequence may have a role. Although little is known, the implication for IGFBP-1 protease activity will further increase the complexity of IGF-I regulation.

2.3.3 The Ternary IGF Binding Protein Complex

The combined molecular weight of the ternary IGF-I: IGFBP-3: ALS complex is approximately 150 kDa and consumes 80-90% of all free IGF-I in serum. Glycosylated IGFBP-3 has an affinity for IGF-I of 2.0×10^{10} L/mol (Martin *et al.,* 1986). The complex stability is affected by pH and ionic strength. Optimal binding occurs at a pH of 5.0 and decreases with increases in pH to physiological pH. High ionic strength enhances the interaction between IGFBP-3 and IGF-I (Holman *et al.,* 1996). ALS is not able to bind IGF alone, but forms high affinity IGF containing complexes only in the presence of IGFBP-3. ALS has an immeasurably low binding affinity for IGFBP-3 alone (Martin *et al.,* 1999). Therefore, it has been postulated that the IGFBP-3: IGF-I complex forms a binding site for ALS. This view has been challenged with the use of patially proteolyzed and unglycosylated IGFBP-3 which show weak interactions with ALS (Lee *et al.,* 1995, Barreca *et al.,* 1995). These conditions have not been demonstrated *in vivo.* With ALS

bound, the half-life of IGF is extended from 10 min in free form and 60-90 min in the binary complex to more than 12 hrs in the ternary complex (Boisclaire *et al.,* 2001). Like the binary complex, the ALS association is dependent upon pH and ionic strength. The affinity constant for ternary complex formation under RT conditions of low ionic strength and slightly acidic pH was approximately 1 x 10^9 L/mol (Baxter *et al.,* 1989). A decrease in affinity to 2.5 x 10^8 L/mol was observed at near physiological conditions of salt, pH and temperature (Holman *et al.,* 1996).

The interactions between IGF-I, IGFBP-3 and ALS in formation of the ternary complex depend on many factors. In formation of the IGFBP-3 binary complex, glycosylation seems to play a minimal role. Comparison between the IGF-I binding affinities of IGFBP-3 produced in *E.coli* (no glycosylation) with that of IGFBP-3 from mammalian origins (glycosylation) result in little or no change in affinity (Conover 1991). Chinese hamster Ovary (CHO) cells expressing an IGFBP-3 N-terminal fragment, IGFBP-3 [1-88], show a large reduction in IGF-I binding activity compared to the intact IGFBP-3 protein. In separate studies, removal of negatively charged amino acid residues within the C-terminus ($Lys^{254}Glu^{254}$ to ArgGly) decreases IGF binding 75-80% (Firth *et al.,* 1998). Domain swapping experiments with IGFBP-1-6 have shown that the carboxy terminal domains of IGFBP-3 are also important for ALS binding to the ternary complex. This has been further mapped to a group of 18 amino acids which are basic and positively charged (Twigg *et al.,* 1998) (Figure 2.6).

ALS contains seven N-glycosylation sites in its primary structure. Structural analysis of ALS has indicated that in the proposed donut conformation of Janosi *et al.,* (1999), the seven N-terminal N-linked sugars are clustered providing a strong pocket of negative charge due to the presence of negatively charged sialic acids. Removal of the sialic acid residues from the glycan chain of ALS results in reduction of affinity for IGFBP-3 binary complexes but do not eliminate complex formation. Independent mutation of the seven N-linked glycosylation sites results in partial reduction in affinity for IGF-I complexes. Complete deglycosylation eliminates all interaction with the IGF-I/IGFBP-3 complex (Janosi *et al.,* 1999a). Thus, the model that exists identifies a positively charged, conserved domain of 18 amino acids present in IGFBP-3 as interacting with two dense pockets of negative charge on ALS. The negatively charged

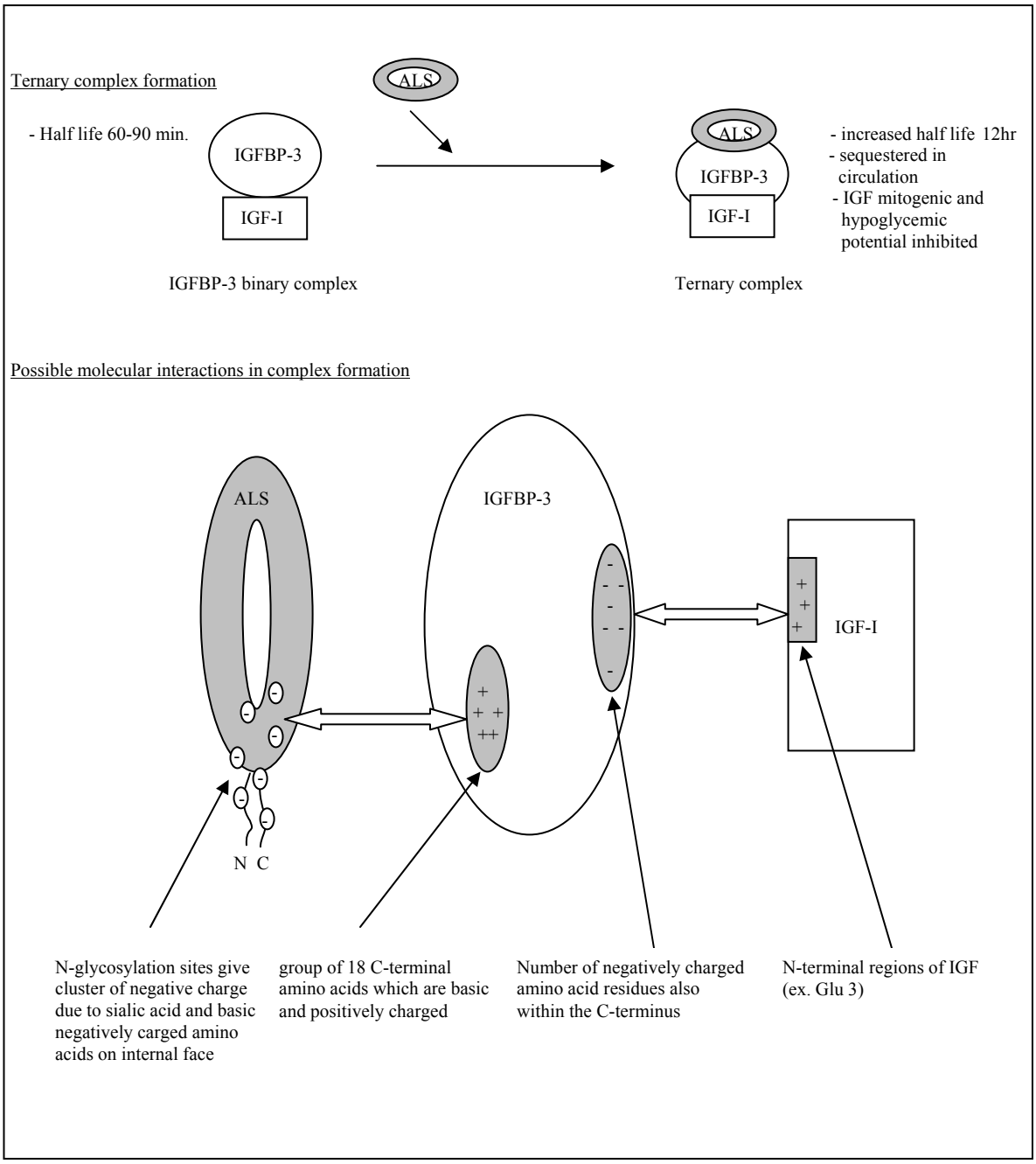


Figure 2.6 Interactions which mediate the formation of the ternary IGFBP complex.

pockets on ALS are due to N-terminal clusters of N-linked sugars as well as basic and negatively charged amino acids located on the internal face of the donut shaped structure of ALS (Janosi *et al.,* 1999b) (Figure 2.6).

IGFBP-3 is susceptible to proteolysis and, as such, exists as a mechanism to modulate IGFBP-3 activity. A variety of metabolic conditions that lead to IGFBP-3 proteolysis include catabolic states such as type-1 and type-2 diabetes and pregnancy. During human pregnancy, a 30 kDa form of IGFBP-3 is found exclusively in serum and is found to have an 8-fold decrease in IGF-I affinity (Binoux *et al.,* 1994). However, levels of circulating IGF ternary complex during pregnancy are normal or elevated, indicating binding affinity may not be lost by the proteolized IGFBP-3. The explanation for this conflicting data is that ALS is acting to increase the affinity of proteolyzed IGFBP-3 for IGF-I by 7-fold to stabilize the formed ternary complex (Gargosky *et al.,* 1991). This idea has been highly debatable and many investigators have shown that complex formation with the 30 kDa IGFBP-3 fragments result in a decreased affinity for IGF-I and therefore an increased release of bioactive IGF-I. Other proteolized forms of IGFBP-3 have also been identified with decreased IGF-I affinity. For example, a 22 kDa plasmin fragment of non-glycosylated IGFBP-3 has a 50-fold reduction in IGF-I affinity (Lalou *et al.,* 1996). A 30 kDa form of rat IGFBP-3 has a 50 fold preferential increase in IGF-II affinity (Lee *et al.,* 1995). At the tissue level, it is postulated that proteolysis may allow release of IGFBP bound to IGF leading to local IGF bioactivity. IGF-I exists in circulation at a concentration of 100 nM (700-800 ng/mL) and in normal conditions exhibits 5-10% of the hypoglycemic potential of insulin. Circulating IGF-I, therefore, if not bound in IGFBP complexes, has the potential to vastly exceed insulin in its hypoglycemic potential. IGFBP-3 and ALS, together in the ternary complex, function to sequester IGF and inhibit its potential activity. Administering potentially lethal hypoglycemic doses of IGF-I together with IGFBP-3 ablates any hypoglycemic effect (Sommer *et al.,* 1991). IGF-I administration, with or without IGFBP-3, form ternary complexes within the first three minutes of treatment (Lewitt *et al.,* 1994) showing that ternary complexes form readily in serum. An illustration of the inhibitory effect of ternary complexes comes from patients with a clinical condition called non-islet-cell tumour hypoglycemia (NICTH). These patients have IGF-II secreting tumours and

experience severe hypoglycemia. In these cases there are large increases in free IGFs and a feedback induced decrease in GH secretion. As a result, ALS expression decreases and IGFBP-3 exists mainly in binary complexes which are able to, presumably, cross capillary barriers. GH or glucocorticoid administration alleviates the hypoglycemic effects through increasing ALS and IGFBP-3 levels and thus clearly indicating the inhibition of IGF activity (Frystyk *et al.,* 1998).

The generation of ALS-null mice has allowed investigators to clarify the involvement of ALS in a manner delineated from other IGF system components. In ALS-null mice, the ternary complex is absent in circulation (Ueki *et al.,* 2000). The ALS-null mice have dramatically reduced concentrations of IGF-I and IGFBP-3 in circulation (62 and 88% decrease, respectively) as compared to wild type mice. Hepatic expression of IGF-I and IGFBP-3 in the ALS-null mice is normal indicating the importance of ALS in maintaining a large accumulation of IGF-I and IGFBP-3 in circulation. In single allele ALS-null mice, ALS secretion is decreased. IGF-I and IGFBP-3 levels were again lower (17% and 40% decrease, respectively) as compared to wild-type. The ALS-null mice showed only a 13% growth deficit when compared to wild type. A significant effect was expected due to the central role of IGF in growth regulation and the decreases in IGF-I and IGFBP-3 levels. In ALS-null mice it was also surprising to see normal levels of insulin and glucose since IGF is known to have a strong hypoglycemic potential. A proposed explanation was in the noted absence of IGF-II. Mammals have high levels of IGF-II. IGF-II has been shown to be a more potent insulin-like effector and it may be plausible that lowered concentrations lead to decreased hypoglycemic effects (Ueki *et al.,* 2000, Boisclaire *et al.,* 2001).

In the ternary complex, IGF is believed to be restricted to the vascular system due to the inability of the ternary complex to traverse the capillary barrier. It may be possible that some crossover may occur since ternary IGF complexes have been identified in a number of extrahepatic fluids such as skin interstitial fluids, mammary lymph and wound fluid (Martin *et al.,* 1999). ALS has a relatively weak affinity for IGFBP-3 and may allow for easy disassociation and subsequent release of IGF-I. However, ALS circulatory concentrations far exceed the required amount for ternary complex formation. It has thus been excluded from discussion relating to mechanisms of escape into extravascular

tissues even though complex dissociation may occur in order for passage out of the circulatory system. Dissociation of ALS from the ternary complex has been shown *in vitro*. Proteoglycan and more specifically, Heparin, mediates the reduction in the affinity of ALS for IGFBP-3, but this has not been shown *in vivo* (Baxter 1990b). Limited IGFBP-3 proteolysis may be expected to also decrease ALS affinity for the IGFBP-3 binary complex. In pregnant women, association constants may not change between proteolized and non-proteolyzed complexes. It remains to be found whether IGFBP-3 proteolysis may induce escape from circulation.

Complexation of IGF-I in ternary complexes also has effects on IGF-I mitogenic potential. This has been shown in a number of studies at the cellular level with much of the work derived from human skin fibroblasts. Co-incubation or pre-incubation of IGFBP-3 and the ratio of IGFBP-3 to IGF-I are very important factors in determining whether the IGFBP-3 effect is inhibitory or stimulatory on IGF mediated mitogenic activities. Co-incubation of equimolar ratios of IGFBP-3 and IGF reversed the mitogenic effects of IGF in human fibroblasts in a dose dependent manner. In contrast, pre-incubation of cells with IGFBP-3 for 8-48 h before adding IGF-I resulted in a potentiation of the subsequent IGF-I effect (De Mellow *et al.,* 1988). IGFBP-3 transfected BALB/c 3T3 fibroblasts, in the presence of serum (IGF-I), also exhibited inhibited cell proliferation. Insulin was not able to override this effect of endogenous IGFBP-3. Insulin, however, was able to override the effect of exogenous IGFBP-3 inhibition (Cohen *et al.,* 1993). A dual mechanism for both IGF-dependent and independent activity was postulated for IGFBP-3 and its effect on cell proliferation. Reports of IGFBP-3 expression being induced by the tumour suppressor, p53, have also implicated IGFBP-3 in the mechanism of programmed cell death. This has been strengthened by results indicating that mutant p53 fails to induce expression of IGFBP-3 in cells exhibiting a loss of apoptotic activity (Buckbinder *et al.,* 1995).

In some cases the concentration of ternary complex positively correlates with growth. In clinical conditions with GH excess or through GH administration, which causes an increase in ternary complex component (ALS and IGFBP-3) concentrations, a concomitant increase in growth response occurs. From these studies there is support for the idea that the ternary complex forms a reservoir from which IGF activity can be made

available. However, this conflicts with the idea that IGF-I must be in free form to elicit its effects on cells. Studies show that it is not necessary to form tight IGF complexes. Ternary complexes containing des(1-3) IGF-I, which binds with seven fold less affinity, are at least as effective as IGF-I (Martin *et al.,* 1999). Studies have furthered the argument of complex activity in wound healing where it was found that IGF:IGFBP-3 applied topically was more effective than IGF-I alone in stimulating wound recovery (Sommer *et al.,* 1991).

The potentiating effects of IGFBP-3 correlate with an increased level of IGF-1R interaction at the cell surface. This may be due to the presence of serine proteases (Canover *et al.,* 1996). The resulting IGFBP-3 fragments, as with other IGFBP fragments, may have decreased binding affinity for IGF-I and result in an increase in free IGF bioactivity at the cell surface. Studies have found that the 30 kDa fragment (lacking the C-terminal third of the protein) purified from rat serum enhances IGF-I stimulated DNA synthesis whereas intact IGFBP-3 blocks stimulation in osteoblasts (Schmid *et al.,* 1991) This induced mitogenic activity is inhibited with the addition of serine protease inhibitors (Angelloz-Nicaud *et al.,* 1995). Similarly the IGFBP-3 plasmin fragment of 22kDa also stimulates PC-3 cell proliferation but it does so in the absence of IGF-I (Angelloz-Nicaud *et al.,* 1998). IGFBP-3 has been indicated to act independent of IGF-I and has unique features to strengthen this supposition including a heparin binding motif and nuclear localization sequence. It has also been shown that IGFBP-3 has its own receptor on cell surfaces which appear to be TGF-β receptors (Leal *et al.,* 1997). Addition of IGF inhibits the binding of IGFBP-3 to these cell surfaces. This further enhances the evidence for IGFBP-3s anti-proliferative effect and indicates this effect independent of IGF-I.

2.4 Metabolic and Mitogenic Modulators of IGF System Component Expression

2.4.1 Insulin

The insulin receptor (IR) is a member of the receptor tyrosine kinase (RTK) superfamily and more specifically, the insulin receptor family, which also includes IGF-

IR. Unlike the EGF and PDGF receptors, where dimerization occurs upon ligand binding, the IR is dimeric even in the absence of ligand. The receptor is a heterotetramer composed of two α and two β chains. The α chains contain most of the extracellular portion of the receptor, a cysteine rich domain and 2 fibronectin type III domains. The β chain contains the transmembrane domain and the cytoplasmic portion of the receptor containing the tyrosine kinase domain. IR ligand binding induces conformational changes leading to autophosphorylation of three tyrosine residues located in the tyrosine kinase domain (White *et al.,* 1994). Phosphorylation of the tyrosine residues in the so-called "activation loop" of RTKs allows a conformational change which gives unrestricted access to ATP and substrates. Without ligand binding the kinase activity is inhibited by the α subunit's influence on receptor conformation and ATP is blocked from entering the kinase active site (Hubbard 1999).

The IR requires interaction with receptor-specific substrates for signal transduction. Phosphotyrosines (pYs) and src homology -2 (SH2) domains of downstream signaling molecules associate through a three amino acid motif on the C-terminal side of the pY residue (Nystrom *et al.,* 1999). The two major IR substrates are: IRS-1 and IRS-2. Other IR substrates critical for insulin signaling are IRS-3, -4, GAB-1 and Shc. Common to IR substrates are N-terminal pleckstrin homology (ph) domains. In IRS (1 to 4) pY binding domains (PBD) mediate interaction with the IR. IR substrates often have a number of pY motifs which act as docking sites for SH2 containing proteins.

Signaling proteins containing SH2 domains have a number of different functions. Some may act as affector molecules with an intrinsic catalytic function while others may act as adaptor proteins. For example, Grb2 is normally pre-bound to the SOS guanidine exchange factor, but when bound, via the SH2 domain, to an IRS-1 pY residue SOS catalyzes the Ras exchange of GTP for GDP and activates Ras (Figure 2.7). Another example is the PI-3K p85 subunit, which after interacting with IRS-1, activates the PI-3K p110 catalytic subunit (Nystrom *et al.,* 1999).

Activation of Ras and PI-3K give rise to phosphorylation cascades. Ras directly activates Raf, a serine/threonine kinase, which in turn phosphorylates and activates MEK. Active MEK then phosphorylates MAP kinase, Erk1, Erk2 and c-Jun NH2-terminal

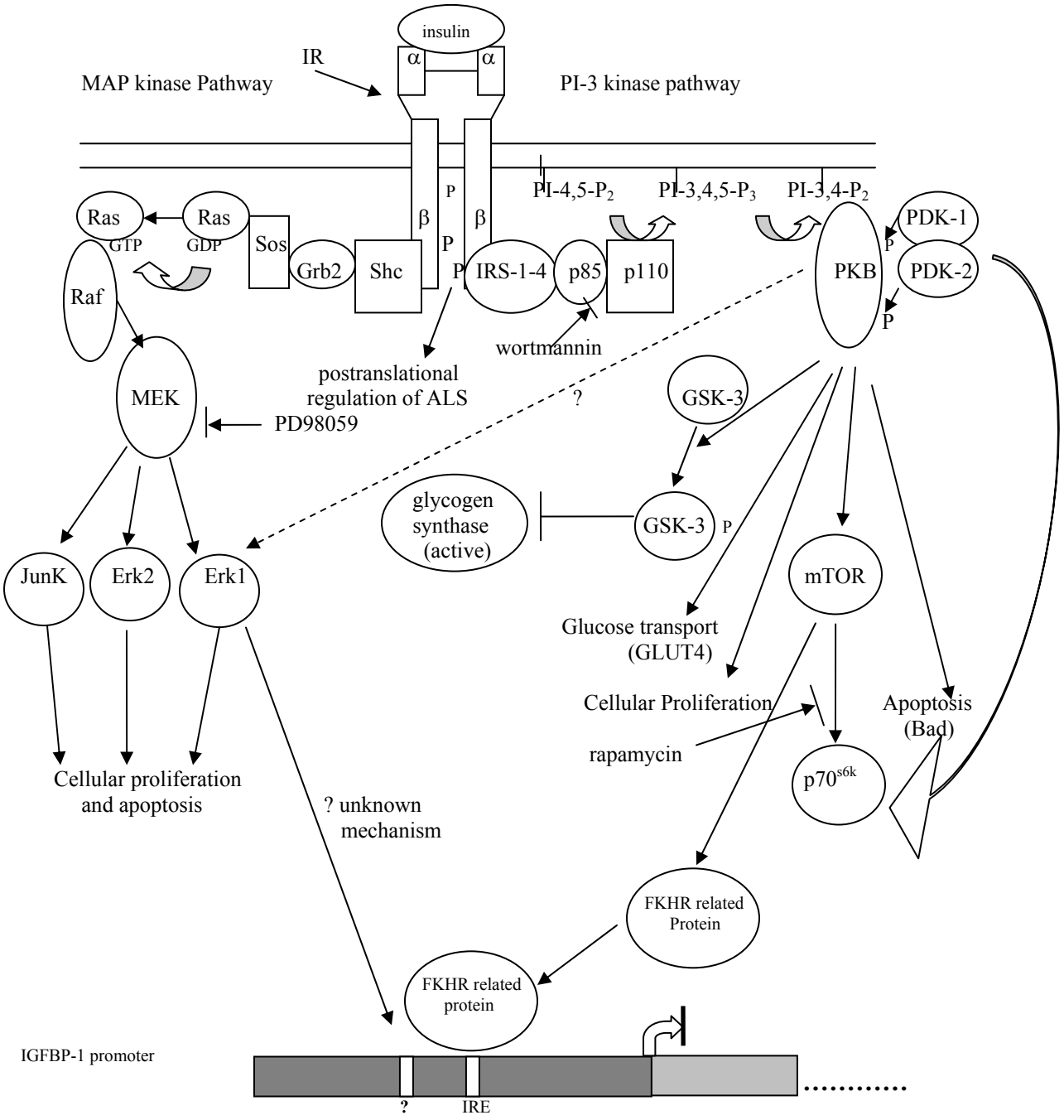


Figure 2.7 Insulin receptor signal transduction and repression of IGFBP-1 gene expression. See text for full details on signaling pathways and relation to IGFBP-1 expression.

Abbreviations: P , Phosphorylation; abbreviations are given in the List of Abbreviations on page xii

Kinase (JNK) which has roles in cellular proliferation and apoptosis (Figure 2.8) (Nystrom *et al.,* 1999). PI-3K phosphorylates inositides on the inner cell membrane and 3-phosphoinositide products. Phosphoinositol-3, 4, 5-triphosphates (PI-3,4,5-P_3) activate phosphoinositide-dependent kinase-1 (PDK-1) and PDK-2. PDK-1 phosphorylates protein kinase B (PKB) on residue Thr308 whereas PDK-2 phosphorylates PKB on residue Ser473 within the PKB regulatory region. Active PKB phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3) which in turn leads to activation of glycogen synthase (Patel *et al.,* 2002). Accumulation of PI-3,4,5-P_3 also gives rise to the activation of S6 protein kinase (S6K or $p70^{s6k}$) through a mechanism dependent on PDK-1 and a kinase termed the mammalian target of rampamycin (mTOR). Activity of mTOR may be dependent on PKB for its activation (Raught *et al.,* 2001). S6K phosphorylates ribosomal S6 protein and may be involved in the insulin regulation of protein translation (Price *et al.,* 1992, Jefferies *et al.,* 1994).

Insulin has been shown to suppress IGFBP-1 expression while stimulating ALS expression. Over expression studies using inhibitors of the PI-3K pathway have shown a possible role for PI-3K, PKB and fork head related protein (FKHR) in the regulation of IGFBP-1 expression by insulin (Figure 2.7) (Cichy *et al.,* 1998, Rena *et al.,* 1999, Guo *et al.,* 1999). FKHR is a member of the FOX(o) transcription factor family and may be activated through phosphorylation by PKB (Rena *et al.,* 1999, Patel *et al.,* 2002). The IGFBP-1 gene contains a thymine rich IRE (TIRE) required for insulin induced repressive IGFBP-1 regulation (Suwanichkul *et al.,* 1994). FHKR-related binding to the TIRE sequence has been shown *in vitro* (Durham *et al.,* 1999). Inhibition of mTOR with rapamycin strongly antagonizes basal insulin repression and glucocorticoid-induced IGFBP-1 expression. Transfection studies utilizing a TIRE-luciferase reporter construct in H4IIE cells show complete abolishment of insulin repression in the presence of rapamycin (Patel *et al.,* 2002).

Although mTOR activity has been shown to be important in the insulin mediated repression of IGFBP-1 expression, a second rapamycin-resistant element is present within the TIRE sequence. The insulin-mimetic agent, peroxovanadium (bpV(phen)), showed IGFBP-1 gene repression independent of PI-3K and S6K. An alternative pathway, independent of PI-3K, may be responsible for repression of IGFBP-1. The

wortmannin inhibition of insulin signaling caused a decrease in ERK activity indicative of PI-3K pathway involvement. However, wortmannin did not abolish the increases in ERK1 activity with bpV(phen)-stimulated cells. Using the MEK inhibitor, PD98059, ERK1 activity did not change. Another protein may be responsible for ERK activation and ERK repression of IGFBP-1 expression (Band *et al.,* 1997).

Insulin does not affect ALS expression at the transcriptional level, but rather affects at the posttranslational level. Insulin increases ALS secretion in the absence of any change in mRNA within primary rat hepatocytes (Dai *et al.,* 1994a, Boisclair *et al.,* 2001). Little is known as to the cell signaling responsible for the insulin effect on ALS expression and secretion.

2.4.2 cAMP

The cAMP biosynthetic pathway is initiated in metabolic conditions having low circulating glucose levels (i.e. exercise or starvation) and released glucagon into circulation. Hepatic cells and adipocytes express β-adrenergic receptors which bind glucagon with high affinity (Figure 2.8). Upon ligand association, a conformational change occurs in the receptor. This change allows the association of a GDP bound G protein composed of two subunits, the α subunit with GDP bound and the β,γ subunit. Once the G protein has associated to the ligand bound receptor the association induces the exchange of a GDP for GTP on the α subunit. The α subunit dissociates and binds a membrane bound adenylate cyclase enzyme. Upon binding to the G protein subunit, adenylate cyclase is activated leading to the conversion of ATP to cAMP, PPi and loss of the Pi from the α subunit bound GTP. The G protein subunits re-associate and are ready for activation once more.

In the liver, the cAMP-responsive signaling pathways play important roles in gene regulation. Correlations in liver gene expression have been made to increases in cAMP levels, modulations in G proteins, adenylate cyclase and protein kinase A (PKA) expression (Diehl *et al.,* 1996, Diehl *et al.,* 1992, Ekanger *et al.,* 1989). Upon stimulation of the adenylate cyclase pathway and biosynthesis of cAMP, transcriptional regulation is mediated by a group of cAMP-responsive nuclear factors through distinct promoter

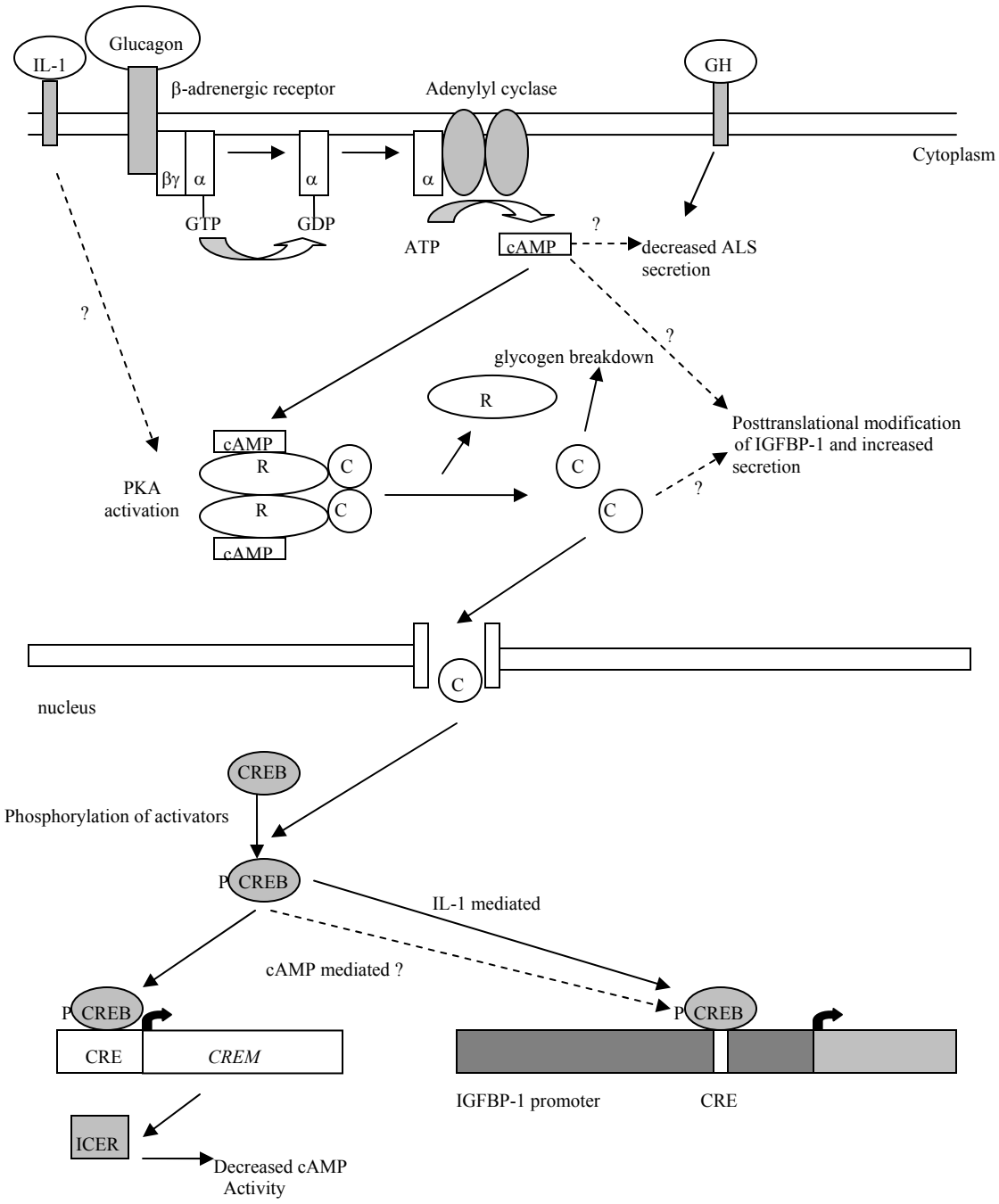


Figure 2.8 cAMP biosynthesis, downstream signaling and regulation of ALS and IGFBP-1 gene expression. Above is a symbolic representation of the information presented in text. For descriptions see text

Abbreviations: C, PKA catalytic subunit; R, PKA regulatory subunit; P, phosphorylation. For remaining abbreviations see List of Abbreviations page xii

responsive sites. These factors are either activators or repressors and contain basic domain/leucine zipper motifs which bind as dimers to cAMP-reponsive elements (CRE). Increased cAMP levels activate the tetrameric PKA which is composed of two regulatory subunits and two catalytic subunits. Once cAMP association to PKA occurs the catalytic subunits are released. Activated PKA regulates glycogen metabolism through phosphorylation of phosphorylase kinase and subsequent phosphorylation of glycogen phosphorylase. Catalytic subunits of PKA also enter the nucleus and phosphorylate nuclear target proteins such as CRE-binding protein (CREB). Another member of the cAMP-responsive nuclear protein family is the inducible cAMP early repressor (ICER) protein which is the product of the *CREM* gene and strongly represses cAMP activity. cAMP treatment of hepatoma cells results in strong CREB phosphorylation and increases in ICER levels (Figure 2.8) (Servillo *et al.,* 1997). Both CREB and CREM have major functions in cAMP mediated responses within hepatocytes affecting metabolic responses such as inhibition of glycogenesis, stimulation of gluconeogenesis through activation of PEPCK as well as effects on differentiation and cell proliferation.

Very little is known as to how IGFBP-1 and ALS are regulated by cAMP. Delhanty *et al.,* (1998b) indicated that cAMP decreases ALS expression, *in vitro,* through the reduction of ALS mRNA in primary hepatocytes. In cAMP promoting states such as acute starvation or nutritional deprivation, serum ALS levels are also decreased (Dai *et al.,* 1994b). Dose-dependent cAMP effects on ALS mRNA levels are only seen in the rhGH stimulated cells indicative of a pre-translational cAMP mediated effect. Basal ALS gene expression exhibited no decrease in expression with increasing cAMP dosage. These results indicate that transcriptional regulation of ALS may depend on the status of GH in the system and cAMP may regulate primarily at a translational or posttranslational level (Delhanty 1998b).

IGFBP-1 expression is stimulated by cAMP at transcriptional and post-transcriptional levels (Unterman *et al.,* 1991). Glucagon has been shown to increase circulatory concentrations of IGFBP-1 in HepG2 cells. Glucagon increased IGFBP-1 protein levels without having any effect on IGFBP-1 mRNA levels. The effect of glucagon on IGFBP-1 expression does not involve a transcriptional level of regulation (Hilding *et al.,* 1993). However, IL-1β, which has also been shown to stimulate IGFBP-1,

appears to effect expression of IGFBP-1 through a PKA/CREB/CRE dependent pathway just as seen for cAMP responsive genes (Frost *et al.,* 2000, Suwanichkul *et al.,* 1993a). These *in vitro* results are conflicting as many *in vivo* studies have shown a glucagon effect on IGFBP-1 mRNA levels (Kachra *et al.,* 1991) (Figure 2.9).

2.4.3 Dexamethasone

Glucocorticoids (GC) function to maintain homeostasis in mammalian organisms through regulation of glucose, fat and protein metabolism. Due to the importance of GCs in the co-ordination of gene expression in many cell types, there is a tight control on its release through the hypothalmo-pituitary-adrenal axis. GC is a adrenocorticoid and occurs naturally but is also produced synthetically as in the case of dexamethasone. As steroid hormones, GCs readily cross cell membranes due to their intrinsic hydrophobic nature. Once in cells, GC binds glucocorticoid receptors (GR). The GR has a modular structure consisting of a DNA binding domain, a ligand binding domain and two activation domains (Beato *et al.,* 1995). The GR exists in its cytosolic unbound form associated in a complex with heat shock protein (HSP) 56 and 90. Upon ligand binding HSP56 and HSP90 dissociate from the GR and the activated GR is translocated to the nucleus where it controls transcription of specific target genes (Figure 2.9) (Tronche *et al.,* 1998).

GR can both activate and repress the transcription of target genes through binding to GC responsive elements (GRE) present in promoters or enhancers. GC affects transcription through direct interaction with basal transcriptional machinery or through crosstalk with other co-factors and transcriptional factors. GC induces expression of several anti-inflammatory proteins such as interleukin (IL)-1 receptor antagonist and IκBα (Clark *et al.,* 2003). GC also activates the transcription of many liver gluconeogenic metabolic enzymes such as PEPCK, glucose-6-phosphatase, tyrosine amino transferase (TAT) and serine dehydratase following dimerization of GR and binding to palindromic GREs. Binding of the active GR to the PEPCK and TAT promoter regions has been shown to involve a N-terminal ligand independent domain and a C-terminal ligand dependent domain which both flank a central domain containing two

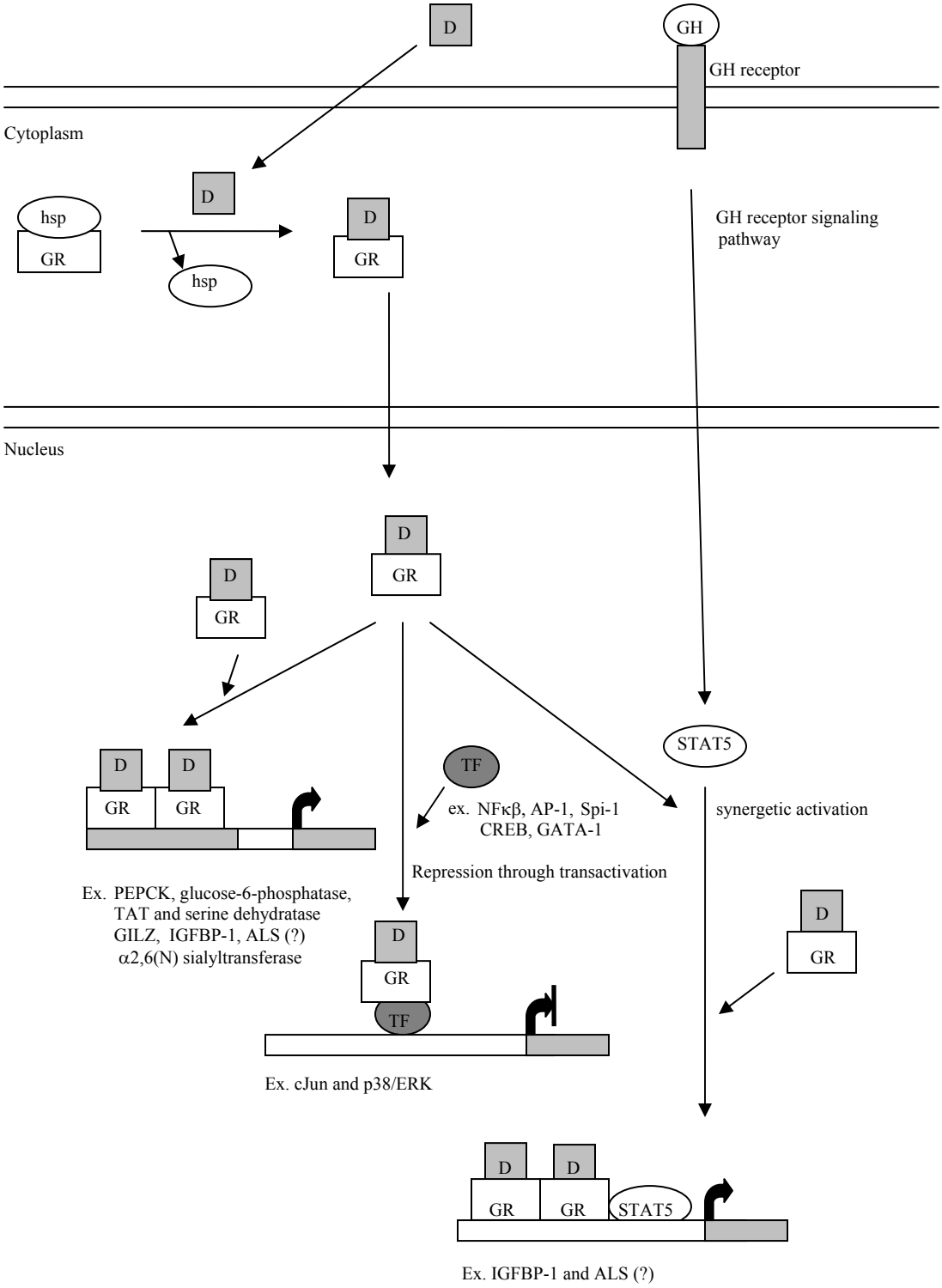


Figure 2.9 Dexamethasone signaling through the glucocorticoid receptor and the regulation of IGFBP-1 and ALS. Above is a symbolic representation of the information presented in text. For descriptions see text.
Abbreviations: see List of Abbreviations page xii

zinc finger motifs utilized for DNA binding and GR dimerization (Groner *et al.,* 2002, Clark *et al.,* 2003). In H4IIE hepatoma cells dexamethasone was found to induce the expression of α2,6(N)-sialyltransferase which catalyze the transfer of activated sialic acids to terminal positions on carbohydrate chains of glycoproteins and glycolipids (Coughlan *et al.,* 1997).

Activated GRs can also repress gene expression through direct repression or through interaction and crosstalk with other proteins. GR can repress transcription when bound to a specific DNA sequence called the negative GRE (nGRE) (Drouin *et al.,* 1993). GR crosstalk with TFs such as NFκβ and AP-1 also leads to direct repression of transcription. This protein-protein interaction has been termed transactivation as association inhibits transcriptional activation while not affecting the binding capacity of the TFs. A similar antagonism exists with other TFs such as CREB, GATA-1 and Spi-1 (Tronche *et al.,* 1998).

Recent evidence suggests that GC induction of glucocorticoid-induced leucine zipper (GILZ) and MAPK phosphatase-1 (MKP-1) protein expression provides crosstalk between the GR and the MAP kinase pathway. In mammalian cells, there are three main MAPK families: ERKs, JNK and p38s. MAP kinase pathways are organized into parallel signaling cascades mediated by sequential phosphorylations. GC induction of GILZ leads to inhibitory actions on MAPK pathway activity through formation of cytoplasmic complexes with Raf and inhibits Raf activity by an unknown mechanism. Association of AP-1 and NFκB directly to responsive genes inhibits induction of gene expression by cJun and p38/ERK. So far, genes inhibited or blocked by GILZ include Fas ligand and IL-2 to date. Little is known about the mechanism of MAPK pathway regulation by MKP-1 but it has been shown that it inhibits p35 and JNK (Clark *et al.,* 2003).

The ability of GCs, such as dexamethasone, to modulate levels of ALS and IGFBP-1 has been shown in rat primary hepatocytes. Physiological amounts of dexamethasone increase H4IIE IGFBP-1 mRNA by 10-fold (Orlowski *et al.,* 1990) and decreases ALS levels through regulation at the transcriptional level. Little is known as to how IGFBP-1 and ALS are regulated and through what mechanism. The relationships between hepatic regulated GH and GR and growth promotion indicates GR physically interacts as a co-activator. GR and STAT5 act in synergy to induce IGFBP-1 expression.

This synergy results in increased transcription rates and growth affects *in vivo* (Tronche *et al.,* 2004). GR is synergistic with other TFs as well such as *jun* homodimers and C/EBPβ (Tronche *et al.,* 1998).

2.4.4 Glucose

The regulation of gene expression via intracellular glucose concentrations has been seen for a number of genes and gene products. These include the stimulation of glucose transportors, glycolytic and lipogenic enzymes (e.g. L-type pyruvate kinase (L-PK), acetyl CoA carboxylase (ACC) and fatty acid synthase) and repression of gluconeogenic pathway enzymes such as PEPCK (Vaulont *et al.,* 2000). In glucose-sensitive tissues, glucose enters cells through specific glucose transporters. In adipocytes and muscle cells the insulin sensitive transporter, GLUT4, is utilized whereas in liver and pancreatic β cells the GLUT2 transporter is utilized. The GLUT2 transporter allows for rapid equilibrium between extracellular and intracellular glucose levels. Further, it has been suggested that the large intracytoplasmic loop of GLUT2 may play a role in signaling due to the hyperexpression of this loop disrupting glucose responsiveness (Guillemain *et al.,* 2000). Once glucose enters liver cells, muscle cells, adipocytes and β cells it is phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase which is the first step in glycolysis (Figure 2.10). G-6-P is utilized in glycolysis, the pentose phosphate pathway and glycogen synthesis in the liver. In muscle cells and adipocytes G-6-P is also used in hexosamine synthesis. Although G-6-P has been implicated as a required molecule for glucose mediated changes in gene expression other downstream intermediates may also be involved.

Utilization of G-6-P in the metabolic pathways has been investigated for possible intermediates involved in glucose mediated changes in gene expression. An intermediate in the non-oxidative branch of the pentose phosphate pathway, xylitol, has been shown to mimic the effects of hyperglycemia on glucose-regulated gene expression (Massilon *et al.,* 1998). In fat and muscle tissue, the hexosamine pathway may be responsible for some of the transcriptional effects of glucose (Sayeski *et al.,* 1996). However, it is widely

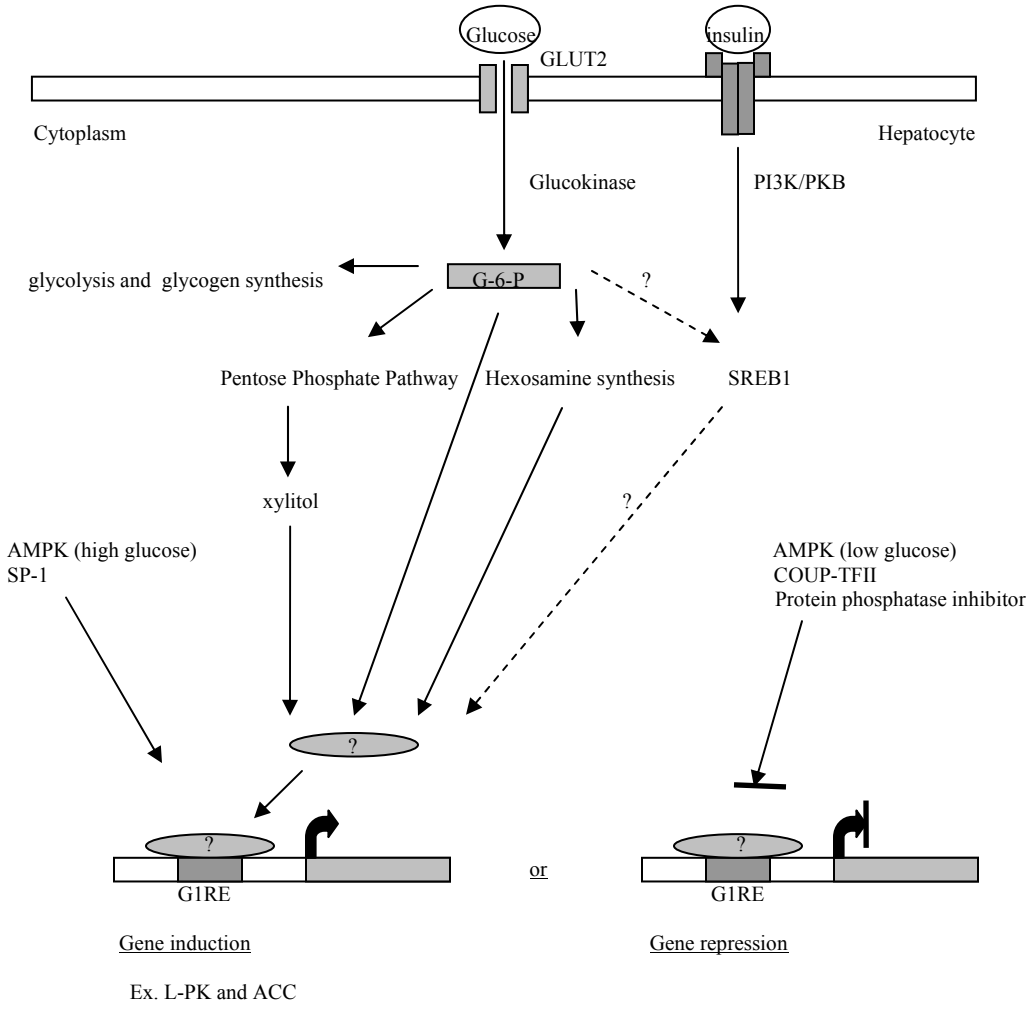


Figure 2.10 Glucose mediated regulation of gene expression in hepatocytes. Above is a symbolic representation of the information presented in text. For descriptions see text.

Abbreviations: see List of Abbreviations on page xii

thought that G-6-P is independently responsible because regulation has been shown to be primarily dependent on the synthesis of G-6-P (Mourrieras *et al.,* 1997).

Much research has implicated phosphorylation/dephosphorylation cycles in the cell signaling and regulation of glucose mediated transcription. Protein phosphatase inhibitors inhibit the glucose induction of L-PK expression (Datta *et al.,* 1999). cAMP dependent protein kinase has also been implicated in regulating glucose dependent signaling (Gourdon *et al.,* 1999). Recently it has been shown that the activation of AMP-activated protein kinase (AMPK) by 5-amino-4-imidazolecarboxamide riboside inhibits the activation of many glucose-regulated genes in primary hepatocytes (Leclerc *et al.,* 1998). In response to cellular changes such as stress, which depletes ATP, AMPK is activated leading to the phosphorylation of many cellular target proteins involved in carbohydrate and fat metabolism. In pancreatic β cells, high glucose leads to the inactivation of AMPK and a subsequent increase in L-PK transcription and vice-versa (da Silva Xavier *et al.,* 2000).

The glucose response element (G1RE) has been defined and is involved in the glucose mediated positive effect on gene transcription (Cuif *et al.,* 1993). The L-PK G1RE consists of two palindromic boxes separated by 5 base pairs, which bind to upstream stimulatory factors (USFs), and an auxiliary site which act synergistically for gene activity (Vaulont *et al.,* 2000). Many G1RE DNA-binding proteins have been reported in addition to USF. An orphan nuclear receptor of the steroid/thyroid hormone class, the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), binds the G1RE *in vitro* and is found in liver extracts. Through G1RE binding COUP-TFII interferes with USF transactivation of the L-PK gene and represses the glucose effect on L-PK transcription (Lou *et al.,* 1999). Other TFs involved are the sterol response element-binding protein 1c (SREBP-1c) (Foretz *et al.,* 1999, Osborne *et al.,* 2000), Sp1 (Daniel *et al.,* 1996a) and pancreatic duodenum homeobox protein (PDX-1) (Macfarlane *et al.,* 1999).

Glucose may have effects on gene expression and a number of possible intermediates may be involved. However, the effects of glucose on components of the IGF system are not yet known. Glucose uptake may have an insulin-independent effect on IGFBP-1 levels (Snyder *et al.,* 1990) but its effect on ALS expression has not been

thoroughly investigated. However, it is well known that nutritional status has an effect on expression of components of the IGF system including IGFBP-1 and ALS. Both hyper- and hypo-glycemia lead to changes in expression of IGF system components.

2.4.5 Growth Hormone

The interactions between GH and the IGF system have been well documented and have been collectively termed the IGF:GH axis. GH has a positive regulatory role on the expression of IGF-I, ALS, IGFBP-3 and IGFBP-1. Therefore, GH levels normally correlate with levels of IGF system components in circulation. Functionally, IGF-I mediates much of the growth promoting activity of GH and controls GH expression through an indirect feedback mechanism through interaction with the pituitary and hypothalmus, the origin of GH and GHRH secretion, respectively. When levels of GH are high, IGF-I functions to inhibit further GH release.

The GH receptor is a member of the cytokine receptor superfamily. The GH receptor contains a single putative membrane spanning domain, a 210 amino acid extracellular region (fibronectin III domain) with conserved pairs of cysteine residues, C-terminal YXXFS–like motifs and two short homologous domains in the intracellular domain entitled box 1 and 2. Box 1 contains a consensus sequence for association with Janus kinase 2 (Jak2) and is required for most GH-stimulated cell functions. Box 2 is less defined but deletion or mutation of these boxes abrogates Jak activation and subsequent proliferative effects on cells (Zhu *et al.,* 2001) (Figure 2.11).

Receptor activation follows the binding of GH to two dimerized GH receptors. With the use of X-ray crystallography technique, it has been suggested that hGH has two receptor binding sites, a high affinity site and a low affinity site. These sites sequentially bind the dimerized GH receptor homodimer and provide the signal required for biological activity (DeVos *et al.,* 1992). The GH receptor lacks an intrinsic tyrosine kinase activity. Activation of signaling relies on the recruitment and/or activation of cytoplasmic tyrosine kinases. Many non-receptor tyrosine kinases have been identified which show an ability to interact with the intracellular motifs of different receptors. These kinases contain SH-1, SH-2 or SH-3 domains (Avraham *et al.,* 2000, Zhu *et al.,* 2001).

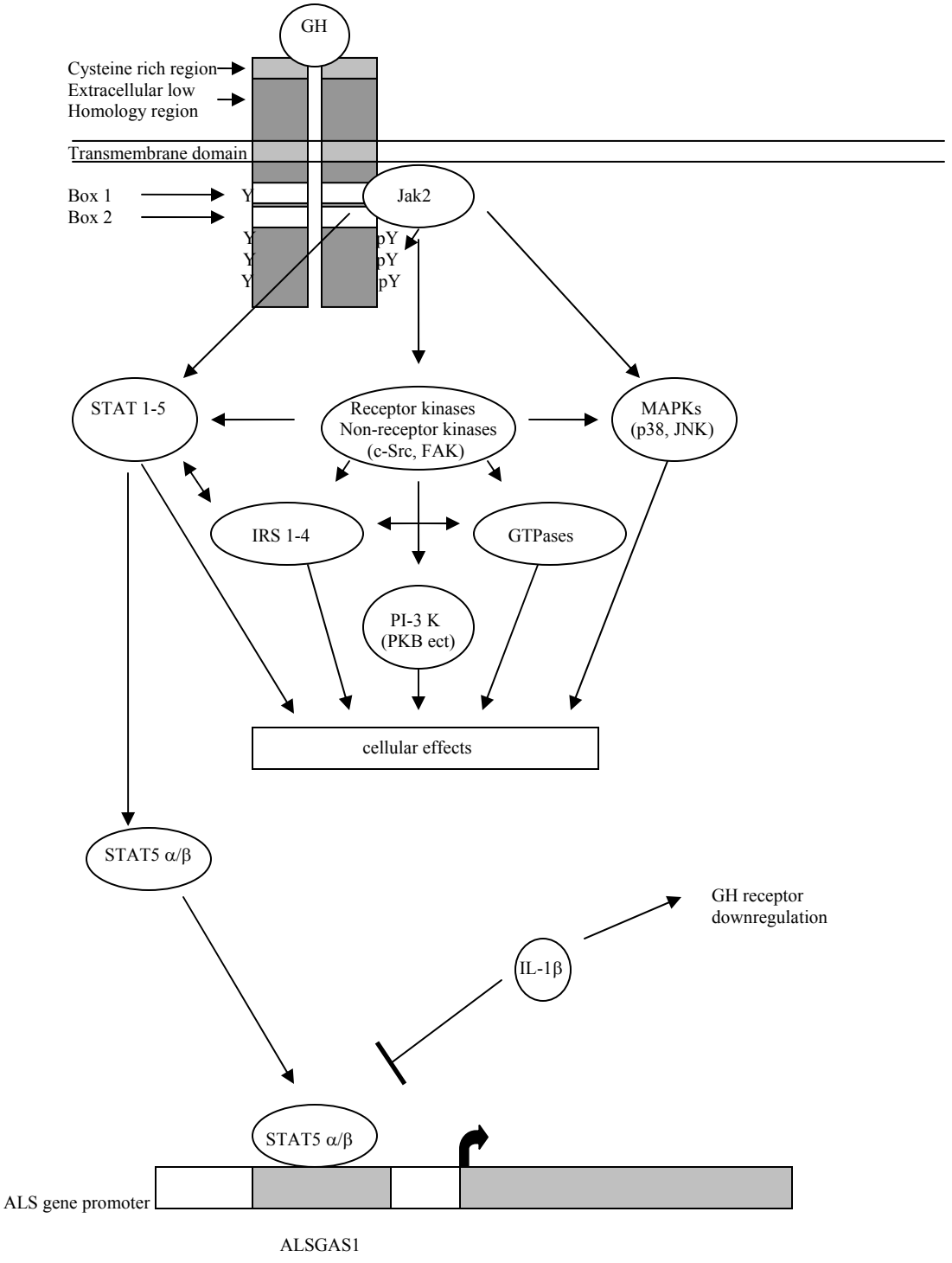


Figure 2.11 The GH signaling pathway and the regulation of ALS expression. Above is a symbolic representation of the information presented in text. For descriptions see text

Partially adapted from Zhu *et al.,* 2001
Abbreviations: pY, phosphotyrosine; for others see List of Abbreviations on page xii

Upon ligand binding and receptor dimerization the Janus kinase, Jak2, binds to the Box 1 sequence which is sufficient for kinase activation. Jak2 phosphorylates sites on the cytoplasmic domain of the GH receptor (Sotiropoulos *et al.,* 1994). Jak2 and the phosphorylated receptor provide docking sites for a number of signaling molecules containing SH-2 or PTB domains. Some signaling molecules which associate to Jak2 are SHC (src homology 2/α collagen-related), IRS, SHP-1 (SH2-containing protein tyrosine kinase phosphatase), p85 subunit of the PI-3 kinase and STAT-1, -3, -5. Other signaling molecules undergo tyrosine phosphorylation and therefore have a role downstream of GH receptor activation such as Raf-1 and IRS-1 which can activate both the MAP kinase and PI-3 kinase pathways (Dominici *et al.,* 2002) (Figure 2.11).

Although it has been shown that a number of signaling pathways are affected by GH mediated receptor signaling, the STAT family has been the most extensively studied and relates to the expression of components of the IGF system. ALS, for example, has been shown to contain a growth hormone-responsive promoter element in the mouse, sheep and human ALS gene (Suwanichkul *et al.,* 2000, Rhoads *et al.,* 2000). A GH responsive promoter between nt -2001 and nt -49 has been shown in rat liver cells. Deletion and mutational analysis of the mouse ALS promoter sequence identified a single γ - interferon activated sequence (GAS) corresponding to nt -633 to -625 which was entitled ALSGAS1 (Figure 2.12). Stimulation of expression is through the binding of Stat5a and Stat5b protein factors to the ALSGAS1 fragment (Ooi *et al.,* 1997, 1998). The ALSGAS1 sequence is highly conserved amongst mouse, rat, sheep and human genes and has been shown to be required for ALS activation by GH (Rhoads *et al.,* 2000, Suwanichkul *et al.,* 2000). GH activation is through the Jak-Stat pathway and its responsiveness is blocked by IL-1β. IL-1β affects responsiveness through the down regulation of the GH receptor or through interference with Stat5 activity. Interference with Stat5 may be mediated by a intracellular suppressor of cytokine signaling (SOCS)-3 which is an inhibitor of the Jak-Stat pathway (Wolf *et al.,* 1996, Boisclair *et al.,* 2000). Interestingly, IL-1β is seen to increase IGFBP-1 expression (Frost *et al.,* 2000). The regulation of IGFBP-1 expression through GH signaling has not been shown and it is possible that IGFBP-1 is regulated independent of GH secretory status (Baxter *et al.,* 1987).

2.5 Experimental Models

2.5.1 Streptozotocin-Induced Diabetic Rats

In 1963, murine tumour studies led to the discovery that streptozotocin (STZ) produced hyperglycemia (Rakieten *et al.,* 1963), and further toxicology studies in dogs and rhesus monkeys demonstrated that STZ had a potent diabetogenic effect (Carter *et al.,* 1971). Since this time streptozotocin has been used in research as a diabetogenic agent in animals. The first rat studies conducted were in 1965 (Evans *et al.,* 1965). The mechanisms of STZ-induced hyperglycemia are considered as follows: STZ causes DNA strand breaks in pancreatic islets and stimulates nuclear poly (ADP- ribose) synthetase, and thus depletes the intracellular NAD+ and NADP+ levels, which inhibits proinsulin synthesis and induces diabetes (Wilson *et al.,* 1988). This is Type I diabetes with no pancreatic insulin production. The study of liver cell expression in STZ-induced diabetic rats has been done by a number of groups. In fact, a group recently has investigated IGFBP and IGF-I liver cell mRNA expression in streptozotocin- induced diabetic rats (Goya *et al.,* 1999).

2.5.2 HepG2 Cell Lines as a Model of Hepatocyte Function and Metabolism

Human hepatoma HepG2 cells (ATTC HB-8065, Javitt *et al.,* 1990) have been used by researchers for over 25 yrs. The cell line was initially established in 1980 as a human hepatocellular carcinoma cell line (Knowles *et al.,*1980). Since that time it has been extensively used in studies investigating hepatocyte function related to metabolism. The study of HepG2 cell line gene regulation by metabolic hormones such as insulin and glucagon has been investigated by numerous researchers (Khamzina *et al.,* 2005, Roesler *et al.,* 1996). Investigation of IGF system component regulation has also been performed in HepG2 cell lines (Frost *et al.,* 2000).

2.5.3 H4IIE Cell Lines as a Model of Hepatocyte Function and Metabolism

Rat hepatoma H4IIE cells (ATTC CRL-1548, Reuber 1961; Pitot *et al.,* 1964) have been used by researchers for over 45 yrs. The cell line was initially established in 1964 as a rat hepatocellular carcinoma cell line (Pitot *et al.* 1964). Since that time it has been extensively used in studies investigating hepatocyte function related to metabolism. The study of H4IIE cell line gene regulation by metabolic hormones such as insulin and glucagon has been investigated by numerous researchers (Patel *et* al., 2002, Crossen *et al* 1997). Investigation of IGF system component regulation has also been performed in H4IIE cell lines (Patel *et al.,* 2002).

3.0 OBJECTIVES

The general objective of this thesis was to investigate the regulation of ALS and IGFBP-1 expression in the liver and in hepatoma cell lines in respect to the hypotheses made in the introduction (section 1.0). To satisfy this objective studies would assess the protein secretion and gene expression of IGFBP-1 and ALS, respectively. The specific objectives are as follows:

1) To identify modulation of IGFBP-1 and ALS expression in rat hepatoma H4IIE cells, time course and dose dependency experiments were performed using a number of candidate modulators of expression including insulin, cAMP, dexamethasone, glucose and GH.

2) To characterize the *in vivo* regulation of liver ALS expression in streptozotocin-treated diabetic rats and streptozotocin-treated diabetic rats with insulin replacement.

3) To investigate the combined effects of cAMP and insulin using methods with the measurement of ALS gene expression as a function of modulator incubation time and order of incubation.

4) To investigate hepatocyte signaling pathways which operate in the regulation of IGFBP-1 secretion and ALS gene expression through the use of modulator co-incubation with MAPK and PI-3K pathway inhibitors.

5) To investigate the function of PKB in the regulation of IGFBP-1 and ALS expression using HepG2 PKB-CA cell lines and comparing expression in these cells to the expression in normal HepG2 cell lines.

4.0 MATERIALS AND METHODS

4.1 Materials

4.1.1 Cell lines

Human hepatoma HepG2 cells (ATTC HB-8065, Knowles *et al.,*1980, Javitt *et al.,* 1990) and rat hepatoma H4IIE cells (ATTC CRL-1548, Reuber 1961; Pitot *et al.,* 1964) were a generous gift from Dr. W. Roesler, Department of Biochemistry, University of Saskatchewan. The HepG2-PKB-CA cell line expresses a constitutively active PKB construct and was a gift from Dr. D. Gupta, Department of Biochemistry, University of Saskatchewan. All cells were grown in Dulbecco's Modified Eagle Medium (DMEM): Nutrient mixture F12 (Ham) (1:1) – with glutamine supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic agent (10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 25 μg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B as Fungizone® Antimycotic in 0.85% saline). Cells were grown on 100 mm tissue culture dishes at 37^{o} C and 5% CO_2 in a humid incubator. Cells were grown 3 to 4 days until approximately 80% confluent. Cell cultures were maintained through passaging using trypsinization with Trypsin-EDTA (1%) (10x solution contains 0.5% Trypsin (2.5% (10X), liquid) and 5.3 mM EDTA * 4Na) in 1x phosphate buffered saline (1xPBS) (Table 4.1) to lift cells off plate. Cells were passaged 1:4 for H4IIE and 1:3 for HepG2 cells. Cells were visualized for confluency (concentration of cells on plate), contamination and cell morphology using a Nikon Phase Contrast-2 microscope. Before cell treatments, confluent cell cultures (80 – 90% coverage of plate) were incubated overnight in the presence of serum-free media (DMEM, 0.2% BSA, 1% antibiotic/antimycotic).

4.1.2 Animals

Male Wistar rats (3 month old, 120-150 g body weight) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) and were used as a source for

mRNA analysis. These animals were kept in an air conditioned environment with a 12 hr light-dark cycle. Animals were fed with liberty a pellet rodent stock which consists of 77% carbohydrate, 16% protein and 7% fat. The research protocols were as approved by the University of Saskatchewan Animal Care Protocol Review Committee as per guidelines of the Canadian Council on Animal Care.

Table 4.1 List of buffers and solutions

Buffer or solution	Components
1 x PBS	7.2 g/L NaCl, 1.48 g/L Na_2HPO_4, 0.43 g/L KH_2PO_4, pH 7.4
10 x MOPS Electrophoresis buffer	0.4 M MOPS, 100 mM sodium acetate, 10 mM EDTA
5 x Run buffer	9 g tris base, 43.2 g glycine, 3 g SDS made to 600 mL with DDH_2O
20 x SSC buffer	3 M NaCl, 0.3 M sodium citrate
50 x Denhardts	1 g ficoll, 1 g polyvinyl pyrrolidine reagent (50x), 5 g BSA (fraction V) dissolved in 100 mL DDH_2O
LB	2.5 g bacto-tryptone, 1.25 g bacto- yeast extract, 2.5 g NaCl in 250 mL DDH_2O
LBA	2.5 g bacto-tryptone, 1.25 g bacto- yeast extract, 2.5 g NaCl, 3.75 g agar in 250 mL DDH_2O
pH 12.5 buffer	1 µL H_2O, 10 µL 5 M NaCl, 8.5 µL 5 M NaOH pH 12.5
pH 7.2 Sodium phosphate buffer	for 100 mL: 1 M Na_2HPO_4 (68.4 mL), 1 M NaH_2PO_4 (31.6 mL)
Protein sample buffer	DDH_2O 3.6 mL, 0.5 M tris-HCl (pH 6.8) 1.0 mL, glycerol 1.6 mL, 10% SDS 1.6 mL, 1% (w/v) bromophenol blue (in water) 0.2 mL
RNA sample Buffer	2.2 (X) formamide, 0.77(X) formaldehyde, 0.44(X) MOPS buffer, 0.44(X) RNA dye (X is sample volume for RNA)
TCM buffer	0.3 M $CaCl_2$, 0.3 M $MgCl_2$, 0.1 M tris-HCl, pH 7.5
TE buffer	10 mM tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0) pH 7.4
Transfer buffer	30.3 g tris base, 144 g glycine, 500 mL methanol to 10 L with DDH_2O
Tris Borate EDTA (TBE) buffer	5x stock solution: 54 g tris base, 27.5 g boric acid, 20 mL of 0.5 M EDTA (pH 8.0) to1 L with DDH_2O

Tritration buffer	100 mM $CaCl_2$, 70 mM $MgCl_2$, 40 mM sodium acetate (pH 5.5)

4.1.3 Plasmids

The rALS /pBSII plasmid construct (Delhanty 1998b) was a generous gift from Dr. Patrick Delhanty from the Kollings Institute of Medical Research, Sydney, New South Wales, Australia. The plasmid was used in transformation of XL-1 *E.coli.*. The RPPO (ribosomal phosphoprotein PO)/pBR322 plasmid vector (Laborda 1992) was generously provided by Dr. W.J. Roesler, Dept. of Biochemistry, University of Saskatchewan. Plasmid were isolated and stored in TE buffer at -20^o C. Bacterial stocks containing vectors were stored at -80^o C in cryogenic vials containing bacterial culture and 15% glycerol.

4.1.4 [I^{125}] -labelled IGF

[I^{125}]-labelled IGF (3.0 x 10^8 cpm/mL) was a generous gift from Dr. van Kessel, Dept. of Animal and Poultry Science, University of Saskatchewan. The samples were stored at -20^o C until required for use in Western ligand blotting.

4.1.5 Chemicals and Enzymes

Enzymes and chemicals used in these experiments are listed in Table 4.2. Addresses of suppliers are given in Table 4.3.

Table 4.2 List of materials used for experiments

Chemicals	**Suppliers**
Acrylamide	Pharmacia
Agarose	Gibco BRL
[α-^{32}P] dCTP	Perkin-Elmer

Agar	Difco
Ammonium persulfate	Sigma
Antibiotic-antimycotic agent	Gibco-BRL
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
Bis acrylamide	Sigma
BSA (fraction V)	Sigma
BSA (RIA grade)	Sigma
Chloroform	BDH
Dexamethasone	Sigma
D- (+) - glucose	Sigma
Diethyl pyrocarbonate (DEPC)	Sigma
DMSO (Dimethyl Sulfoxide)	Gibco BRL
DNase-free pancreatic RNase A	Sigma
Dulbecco's modified eagle medium (DMEM): Nutrient mixture F12 (Ham) (1:1) – with glutamine	Gibco-BRL
Ethidium bromide	Bio-Rad
Fetal bovine serum	Gibco-BRL
Ficoll	Sigma
Folin reagent	Sigma
Formaldehyde	EM Science
Formamide	Sigma
Sephadex G-25 and sephadex G-50	Amersham/Pharmacia
Gene screen plus hybridization transfer membrane	NEN Life Sciences
Growth hormone	Sigma
Clinistix - Glucose strips	Bayer
Glycine	EM Science
Insulin	Sigma
Kodak X-OMAT 5 X-ray film	Kodak
KpnI	New England Biolabs
Lysozyme	Sigma
MOPS	Sigma
NaH_2PO_4	BDH
Na_2HPO_4	EM Science

NaK- tartrate	Sigma
N6,2'-O-dibutyryladenosine 3',5'-cyclic Monophosphate	Sigma
PD98059	Calbiochem
PEG 8000	BDH
Polyvinyl pyrrolidine reagent (50x)	Sigma
Pork ILETIN II NPH insulin isophane	Eli Lilly
Prime-A-Gene system kit	Promega
protease inhibitor cocktail	Sigma
Pronase	Sigma
PstI	New England Biolabs
RNA	Sigma
SacI	New England Biolabs
Salmon sperm DNA	Sigma
Sodium citrate	EM Science
Sodium dodecyl Sulfate.	Schwartz/Mann Biotech (ICN)
Somnotol	MTC pharmaceuticals
Streptozoticin	Sigma
Tetracycline	Sigma
Tissue culture dish	Falcon
Transblot nitrocellulose transfer membrane	Bio-Rad
TriZol reagent	Invitrogen
Trypsin-EDTA	Gibco-BRL
Wortmannin	Sigma
3M paper	Whatman
Transblot nitrocellulose transfer membrane	Bio-Rad

Table 4.3 Addresses of suppliers

Supplier	**Address**
Gibco-BRL	Gibco Life Technologies, Inc. Burlington, Ontario, Canada
Fisher	Fisher Scientific, Winnipeg, Manitoba,
MTC pharmaceuticals	MTC Pharmaceuticals, Cambridge,

	Ontario
Sigma	Sigma Chemicals Co., St.Louis, Missouri, USA
BDH	British Drug House, Toronto, Ontario,
EM science	Darmstadt, Germany
Amerisham/Pharmacia	Amersham Pharmacia Biotech. Baie d'Urfe, Quebec,
Bio-Rad	Bio-Rad Laboratories, Mississauga, Ontario, Canada
NEN life sciences	New England Nuclear, Dupont Canada Inc., Missisauga, Ontario, Canada
VWR	VWR Scientific Inc. West Chester, P.A. USA
Calbiochem	Calbiochem-Novabiochem Corp., La Jolla, CA, USA
Perkin Elmer	Perkin Elmer, Wellesley, MA, USA
Eli Lilly	Eli Lilly and Co., Indianapolis, IN, USA
Invitrogen	Invitrogen Canada Inc. Burlington, Ontario
BRL	Gaithersburg, MD, USA

4.1.6 Apparatus

Table 4.4 List of apparatus used

Apparatus	**Manufacturer and Usage**
Laminar flow hoods	The Forma Biological safety cabinet was used for all work with mammalian cells.
Spectrophotometer	The Bio-Rad Smart Spec was used for determination of protein, RNA and DNA concentrations as well as sample purities
Centrifuges	Centrifugation of large volumes (>10 ml) under 65,000 x g were performed using the RC5C (Sorvall) centrifuge with GSA and SS34 rotors. The eppendorf 5402 centrifuge was used for small volume samples.
Electrophoresis units	For small DNA gels to visualize DNA the Bio- Rad mini-sub cell GT was used. For the running of formaldehyde gels the BRL horizontal system for submerged electrophoresis was used. For SDS-PAGE the Bio-Rad mini-protean 3 gel electrophoresis system was used.
Transfer electrophoresis	For the transfer of protein from SDS PAGE gels to nitrocellulose the Bio-Rad trans-blot system was used.
Freeze dryer	Labonco Freeze dry system and stoppering tray dryer were utilized for

	lyophilization of protein samples
-80 ^{o}C Freezer	Forma Bio-freezer (Forma scientific) was utilized for storage of protein and RNA products
UV illumination box	For visualization of ethidium bromide stained DNA and RNA gels the Transilluminator LM-20E (VWR scientific) was used.
Crosslinker	The UV stratalinker 1800 (statagene) was utilized on all Northern blots
Film developer	The KODAK X-OMAT 1000A film processor was used in the developing of autradiograph x-ray film
Gel Doc system	Bio-Rad Gel Documentation system was utilized along with Quantity One software to observe autoradiogaphs and document them as well as take densitometry readings for all western-ligand and Northern blots.
Microscope	Nikon Phase Contrast-2 microscope was used to observe hepatoma cell growth and morphology
pH meter	pH of all reagents were measured by Accumet pH meter 915 (Fisher)
Water purification	Preparation of double distilled water was through Mega-Pure^{TM} System (MP-6A, Corning Co.)

4.2 Methods

4.2.1 Treatment Conditions for HepG2 and H4IIE Cells

The experimental conditions for isolation of mRNA, protein and secreted protein from HepG2 and H4IIE cell cultures are indicated below. The concentrations of all modulators as added to serum free media and the times of incubation are included for each experiment and are also found in figure legends for respective experiments.

For time course experiments, insulin (10 nM), N 6,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate (db-cAMP) (0.5 mM), dexamethasone (1 μM), growth hormone (GH) (100 nM) and glucose (5.5 mM) were added to cell culture media and incubated for 24-36 hr. The cells or media were collected at 0, 4, 6, 12, 24 and 36 hr.

The concentrations of various modulators used for experiments on the expression of ALS mRNA and IGFBP-1 protein were: insulin (0, 0.1, 1.0, 10, 100, 1000 nM); db-cAMP (ALS) (0, 0.2, 0.5, 2, 4.4, 10 mM); db-cAMP (IGFBP-1) (0, 0.1, 0.25, 0.5, 1.5, 2.5mM); dexamethasone (0, 0.1, 0.25, 0.5, 1.5, 2.5 μM); GH (0, 50, 100, 250, 500, 1000 nM); glucose (0, 5, 25, 50, 100, 200 mM) (above the 17.7 mM which is found in cell growth media). All mRNA related experiments (ALS) had 12 hr incubations while protein experiments (IGFBP-1) had 6 hr incubations.

In the study of IGFBP-1 and ALS expression in relation to modulators and inhibitors of PI-3K and MAP kinase pathways concentrations of modulators were as shown with time course experiments above. Incubation times were 12 and 6 hr with ALS and IGFBP-1 expression, respectively. The pathway inhibitors used were added to culture media 30 min prior to addition of modulators. The concentration of wortmannin and PD98059 were 0.2 mM and 10 μM, respectively. After the pre-incubation, fresh serum free media was added including modulator and pathway inhibitor.

For insulin:cAMP factorial study on ALS expression, 0.5 mM cAMP and 10nM insulin were used with the following ten groups: 1) no treatment, 6 hr; 2) cAMP, 6 hr; 3) insulin, 6 hr; 4) cAMP + insulin, 6 hr; 5) insulin, 12 hr; 6) insulin, 6 hr, then cAMP, 6 hr; 7) cAMP 30 min then insulin, 6 hr; 8) insulin 6 hr then decant off media and add fresh media no modulator, 6 hr; 9) insulin, 6 hr then decant off media and add fresh media with insulin, 6 hr; 10) insulin 6 hr then decant off media and add fresh media with cAMP, 6 hr.

4.2.2 Animal Maintenance, Treatments and Tissue Excision

Male Wistar rats (3 month old, 120-150 g body weight) were used for determining ALS gene expression in liver. Rats were grouped as: 1) Control untreated (Citrate buffer), 2) Streptozotocin (STZ)-induced diabetic and 3) Insulin treated STZ-induced diabetic (Roesler *et al.,* 1987). The rats were allowed to adjust to the local environment. The rats in groups 2 and 3 were made diabetic with a single i.p. injection of STZ (80 mg/kg BW) dissolved in 100 mM citrate (pH 4.5). Control rats (group 1) were injected with the same volume of citrate buffer. The animals were monitored for glucose levels in urine using Clinistix® glucose detection strips. After a period of 5-7 days, all STZ-treated animals were deemed hyperglycemic according to urine glucose levels as indicated on glucose detection strips (maroon /purple). Insulin was subcutaneously injected at a dosage of 2 IU into diabetic group 3 animals once every day for a seven day period. After this period, all animals were killed using Somnotol (1 ml/kg BW rat) and liver tissue removed. Livers tissue was immersed in an equal volume of TriZol and homogenated using a Brinkman polytron. TriZol suspended primary homogenate samples were left to incubate 10 min at RT and mRNA was extracted or frozen for later processing at -80^{o} C.

4.2.3 rALS Fragment Preparation from prALS/BSII Plasmid Construct

The BSII plasmid construct was solubilized in TE buffer, quantified and concentrated by adding 3 M sodium acetate (pH 5.5) (1/10 volume) and isopropanol (7/10 volume) at -20^{o} C for 2 hr. The mixture was centrifuged at 14000 rpm (14900 x g) for 20 min. The pellet was washed with 70% ethanol, dried and reconstituted in TE buffer. The isolated prALS/BSII plasmid construct was utilized in transformation of competent XL-1 MRF^{-} *E.coli* cells.

In production of competent XL-1 MRF^{-} *E.coli* cells a single XL-1 MRF^{-} *E.coli* colony was selected from LBA containing tetracycline (12.5 μg/ml). The positive *E.coli* was then re-cultured in 10 mL LB by shaking 37^{o} C incubator with selective pressure and re-inoculated into 250 mL LB until the OD_{600} reached 0.45 to 0.55 A. XL-1 MRF^{-} *E.coli* were chilled on ice water for 2 hr and collected by centrifugation at 6000 rpm (5800 x g 15 min in GSA rotor). Cells were resuspended in 10 mL tritration buffer and further diluted to 250 mL with the same buffer and incubated on ice for 1 hr. Cells were centrifuged at 1800 x g for 10 min and then gently resuspended in 25 mL ice cold tritration buffer. For long term storage 140 μL DMSO was added per 4 mL cell suspension gently swirled and placed on ice for 15 min. An additional 140 μL DMSO was then added to 4 mL cell suspension, gently mixed and quickly aliquoted into chilled cryotubes. The tubes were quickly frozen in liquid nitrogen and stored at -80^{o} C.

For transformation, the XL-1 competent *E.coli* cells were thawed and the rALS/BSII (1 μL = 100 ng) was added to 19 μL TCM buffer (Table 4.3) and left on ice for 20 min. The sample mixture was then heat shocked at 42^{o} C for 90 sec and put on ice for 5 min. The sample was left at RT for 5 min and 250 mL LB was added. The mixture was incubated at 37 ^{o} C for 1 hr. The culture was plated on to LBA with ampicillin (50 μg / mL) and incubated overnight at 37^{o} C. Transformed colonies were selected the following day.

Plasmid was isolated using the cleared lysate method of plasmid isolation (Clewell *et al.,* 1969). Transformed cells were grown in a 100 ml LB (50 μg/ml ampicillin). The medium was inoculated and shaken at 37^{o} C for 14 to 16 hr. The cells

were pelleted for 5 min at 5000 rpm (4100 x g) at 4 ^{o} C using GSA rotor. The supernatant was poured off and the pellet was washed with 50 mL of 50 mM tris-HCl pH 7.5, 2 mM EDTA. The cells were re-pelleted and resuspended in 1.4 mL 50 mM tris-HCl pH 8.0 in 15 mL Oakridge tubes. For cell lysis, 500 µL of lysozyme solution (10 mg/mL) was added and the mixture was vortexed and left on ice for 10 min. Six hundred µL of 0.5 M EDTA was added to the lysis mixture and again left on ice for 10 min. Finally, 100 µL 2% Triton X-100 was added and left for 60 min on ice. The cell debris was removed by centrifugation (60 min, 8000 rpm, 7600 x g, 4^{o} C, SS34 rotor). The supernatant was then extracted with equal volume of phenol and chloroform. At this stage, 25 µL of 5 M NaCl was added per mL of the supernatant and precipitation was carried out with 0.8 volume of isopropanol at -20^{o} C for 30 min. After 5 min centrifugation (7000 rpm, 5800 x g, at 4^{o} C in SS34 rotor), the supernatant was decanted and the pellet was washed with 80% ethanol and dissolved in 500 µL of TE buffer. The plasmid mixture was then digested with 2.5 µL of DNase-free pancreatic RNase A (10 mg/ml) at 37^{o} C for 1 hr. The DNA was subsequently precipitated with 150 µL of 5 M NaCl and 200 µL 30% PEG overnight on ice. The pellet obtained after centrifugation (20 min, 10,000 rpm, 12000 x g, 4^{o}C, SS34 rotor) was re-dissolved in 300 µL of TE containing 0.5% SDS and transferred to 1.5 mL eppendorf tubes . Proteins were then removed through pronase treatment (3 mL, 20 mg/mL) at 37 ^{o} C for 30 min. Denaturing of the bacterial chromosomal DNA was achieved with 10 min incubation at RT with equal volume of freshly prepared pH 12.5 buffer (Table 4.3). The alkaline reaction mixture was neutralized with 30 µL of 1 M Tris-HCl (pH 7.5) for 5 min on ice. Sodium acetate 3 M (1/10th volume) was added before the DNA was extracted once with equal volume of phenol and chloroform. Ethanol (2.5 volumes) was added for precipitation (2 hr at -20^{o} C). Finally the DNA was pelleted (15 min, 11600 x g), washed twice with 70% ethanol, dried, and resuspended in 200 µL TE. The OD_{260} was taken to determine plasmid concentration (for double stranded DNA: 20 O.D. = 1mg/mL)

To obtain the rALS fragment from BSII plasmid construct the plasmid was digested as follows: 33.75 µL distilled water, 5 µL 10 x NEB buffer #1, 5 µL BSA (10x), 2.5 µL DNA sample (30 ug), 2.5 µL KpnI, 1.25 µL SacI. The reaction was left 7 to 9 hr in 37^{o} C water bath. Agarose gels were poured (0.8% agarose (w/v) in TBE). Digested DNA

samples were loaded on to gel and electrophoresis was carried out at 100 V for approximately 1 hr. The bands of interest were located using UV lamp and the slice of agarose containing the fragment was taken and placed into a 1.5 mL microcentrifuge tube. Gel slices were covered with phenol and stored at -80^{o} C. The phenol immersed gel was thawed and DNA was purified from gel using the phenol-freeze-fraction method. In this method the sample was frozen again at -80 ^{o} C and thawed once more at 37 ^{o} C. This freeze thaw cycle was repeated twice more followed by the addition of 50 µL TE and freezing again at -80^{o} C for 15 min. The samples were then centrifuged at 14000 rpm (14900 x g) for 20 min and upper aqueous layer was transferred to fresh tubes. The aqueous phase was extracted once with chloroform followed by precipitation with 1/10 volume of sodium acetate (3M, pH 5.2) and 2.5 volume of ethanol for 30 min at -20^{o} C. The sample was then centrifuged at 12000 rpm (12600 x g) for 15 min, the pellet was washed with 70% ethanol and solubilized in TE.

4.2.4 ^{32}P-labelling of rALS and RPPO Probes

The purification of RPPO fragment was carried out by the same procedure as outlined for rALS. The digestion of the pBR322 plasmid construct was carried out overnight at 37^{o} C in a reaction mixture containing: 36 µL distilled water, 5 µL 10 x NEB buffer #3, 5 µL BSA (10 x), 2.5 µL DNA sample and 1.5 µL pstI.

The RPPO and rALS fragment probes for Northern analysis were labeled using random priming with the Prime-A-Gene system kit (Promega). 30 ng of purified rALS and RPPO fragment was used to prepare the random labeled probe with denaturing through boiling before addition to reaction mixture. The reaction mixture consists of the following: 5 µL (30 ng) rALS or RPPO (cDNA), 2.5 µL 10 x labelling buffer, 3.0 µL dNTPs (1.0 µL of each of 10 mM stock), 5.0 µL (50 µCi) [α-^{32}P] dCTP, 2.0 µL (5 units) DNA polymerase, Klenow and 32.5 µL of nuclease-free water. The priming reaction was allowed to continue for a period of 4-6 hr. The DNA was labeled to an approximate specific activity of 1.0 x 10^{8} cpm/µg of DNA. The unincorporated radionucleotide was removed using a 1 mL Sephadex G-50 spin column. The purified probe was then boiled for 10 min and chilled for 5 min on ice and used directly for Northern hybridization.

4.2.5 Total RNA Isolation from Rat Liver Tissue and H4IIE and HepG2 Cell Culture

The various plastic containers and apparatus used were rinsed with DEPC treated water before use in these protocols. In addition, the glassware and metal tools used were autoclaved before use.

TriZol suspended rat liver tissue primary homogenate samples were thawed at RT and 500 µL was transferred to 1.5 mL microcentrifuge tube. To this, 500 µL TriZol was added, mixed gently and incubated at RT for 10 min. To this mix, 200 µL of chloroform was added and gently mixed and incubated 2-3 min at RT. The mixture was centrifuged (14900 x g, 4^{o} C, 15 min) and the aqueous phase was transferred to a 1.5 mL microcentrifuge tube containing 500 µL isopropanol. The mixture was then precipitated at -20^{o} C for 1-2 hr and centrifuged (14900 x g, 4^{o} C, 30 min). The supernatant was then discarded and the pellet was washed with 70% ethanol in DEPC treated water. The pellet was allowed to air dry and was resuspended in 40% formamide (DEPC water) and stored at -80^{o} C.

For total RNA isolation from H4IIE and HepG2 cells, confluent cell monolayer plates were placed on ice, conditioned media was removed and cell monolayer was washed 2 times with 1 x PBS (5 mL). After complete removal of wash solution, 1 mL TriZol reagent was added to the plates and was left for 1-3 min. The cells were pooled with the aid of a rubber policeman and then transferred into a 1.5 mL microcentrifuge tube. These samples were stored at -80^{o} C. Further processing was as seen above for rat liver tissue primary homogenate samples.

4.2.6 Protein Isolation from H4IIE, HepG2 and HepG2-PKB-CA Conditioned Media

Protein isolation in all experiments was performed using an identical protocol. Following cell treatments, conditioned media was removed (10 mL) with the addition of protease inhibitor cocktail (10 µL, 1:1000) and was immediately frozen at -80^{o} C. The media samples were thawed on ice and applied to a Sephadex G-25 (fine) desalting column. The protein was eluted with 50 mM Tris-HCl buffer (pH 8.2) and the protein

fraction was identified by measuring absorption at OD_{280}. These fractions were pooled and lyophilized in a freeze dry system. The lyophilized protein powder was reconstituted in water with a concentration factor of 80-100 times and stored at -80^{o} C.

4.2.7 Northern Analysis

Formaldehyde gel electrophoresis was performed using a formaldehyde gel with the following components: Agarose (1% in DEPC treated water), 2.0 g; 10 x MOPS electrophoresis buffer, 20 mL; formalahyde, 35 mL; water (DEPC treated), 145 mL. The gel was poured into a 14 cm x 11cm gel tray and allowed to polymerize.

30-50 μg of RNA sample was mixed in RNA sample buffer (Table 4.1). This RNA denaturing mixture was denatured at 65^{o} C for 10 min and then chilled on ice for 5 min. The gel was placed in an electrophoresis unit and 10x MOPS electrophoresis buffer (Table 4.1) was added to immerse the gel. The sample was then loaded onto the gel based on equal RNA concentrations and was electrophoresed at 2 V/cm gel. The gel was run for approximately 3 hr and put aside for gel transfer protocol. In all cases, a gel was run for assurance of equal loading in each gel and subsequent transfer. These gels were made through addition of 1 μL ethidium bromide per denatured RNA sample volume. Gels were run as usual and total RNA was visualized using UV illumination.

The downward capillary blotting method was performed to transfer RNA to nylon membrane. In this method, the prepared gel was washed for 45 min in DEPC-treated water (repeated 3 times) to remove formaldehyde from the gel. The well portion of the gel was cut off to aid in an even transfer to the Gene screen Plus hybridization transfer nylon membrane. The nylon membrane and three 3M paper (Whatman) pieces were cut with the same dimensions as the gel size and briefly wetted in 10x SSC buffer (Table 4.1). In the preparation of the transfer apparatus, two pieces of 3M paper were cut as wicks long enough to soak in transfer buffer on either side. The wick paper was placed on the center of a square glass plate which was placed upon a large glass container containing 10 x SSC solution. Each of the wick ends were immersed in 10 x SSC. The gel was placed on top of the wick paper followed by the transfer membrane and then the 3 pieces of 3M paper. A small stack (2-3 inches) of paper towel was stacked on top of

3M paper and then a glass plate was stacked on the paper towel and a centered weight was placed on top. The entire apparatus was covered in a plastic wrap to avoid evaporation and was left to transfer overnight. After completion, the membrane was washed in 5 x SSC buffer for 5 min. Transferred RNA was crosslinked on autocrosslink mode using Stratalinker and stored between two wet membrane papers at 4^{o} C.

For the hybridization of probe and the detection of mRNA of interest the crosslinked membrane was placed inside a hybridization bottle and was incubated with prehybridization solution at 50^{o} C in a hybridization oven for 12 to 16 hr. The components of the prehybridization solution for one membrane hybridization are as follows: formamide, 1.0 mL; 50 x Denhardts, 2.0 mL; 20 x SSC, 5.0 mL; 20% SDS, 0.5 mL; 0.5 M EDTA, 0.04 mL; sodium phosphate buffer (1M pH 7.2), 1.0 mL; carrier RNA (10 μg/uL), 0.2 mL; salmon sperm DNA (20 μg/uL), 0.4 mL; water (DEPC treated), 11.2 mL. Following prehybridization the prepared radioactive probe was added to the prehybridization solution and hybridization was carried out for 16 hr at 50^{o} C with gentle mixing. After the hybridization period was finished the labeled blot was washed sequentially as follows with constant readings for background radioactivity and discernment for the specific formation of bands of activity representing the desired mRNA species: 1) 2 x SSC, 0.1% SDS (3 x 5 min, RT), 2) 0.2 x SSC, 0.1% SDS (2 x 10 min, RT), 3) 0.2 x SSC, 0.1% SDS (3 x 10 min, 50^{o} C), 4) 0.1 x SSC, 0.1% SDS (2 x 15 min, 50^{o} C), 5) 0.1 x SSC, 0.1% SDS (1 x 10min, 55^{o} C). After removing background signals, the membrane was wrapped in Saran Wrap and exposed to X-ray film at -80^{o} C for various amounts of time depending on the strength of radioactive signal. X-ray film was developed using a Kodak film processor. The autoradiograms were quantified using the Gel Doc System (Bio-Rad)

4.2.8 Western Ligand Analysis

The Lowry method was chosen for determination of all protein samples due to the predicted lower concentrations of protein which would be secreted into the media. An alkaline solution (30 g Na_2CO_3, 6 g NaOH dissolved in 1.5 L distilled water), NaK tartrate solution (1 g / 100 mL) and Folin reagent (1:1 with water) were made. To make

the alkaline reagent, 0.15 g $CuSO_4$ was added to 10 mL NaK tartrate solution and was mixed until dissolved. From the $CuSO_4$ / NaK tartrate solution 1 mL was transferred to 50 mL alkaline solution and mixed. The alkaline reagent (1.5 mL) was added to 308 µL of the test solution, was gently mixed and allowed to stand at RT for 10 min. 154 µL Folin reagent (1:1) was then added and allowed to stand at RT for an additional 10 min. The absorption was then read against the appropriate blank at OD_{600}. A protein standard curve was done using BSA (0.2 mg/mL) in the range of 0-200 µg BSA and protein was determined against this curve.

Determined protein samples were added at 30-50 µg and mixed with protein sample buffer (5:1 ratio of sample volume to buffer volume), were boiled 3-4 min and were added in equivalent concentrations to gel for electrophoresis. The composition of separating gel was as follows: DDH_2O, 3.35 mL; 1.5 M Tris-HCl, pH 8.8, 2.5 mL; acrylamide/Bis, 4.0 mL;10% SDS, 100 µL; 10% ammonium persulfate, 50 µL; TEMED, 5 µL. The composition of the stacking gel was as follows: DDH_2O, 6.1 mL; 0.5 M Tris-HCl, pH 8.8, 2.5 mL; acrylamide/bis, 1.3 mL; 10% SDS, 100 µL; 10% ammonium persulfate, 50 µL; TEMED, 10 µL. The SDS PAGE was run at a constant voltage of 100 V for 2-3 hr in 1 x running buffer (diluted 5 x running buffer). Upon completion of electrophoresis, the gel was removed from glass plates and allowed to soak in transfer buffer at 4^oC for 20 min.

For protein transfer to nitrocellulose, 4 equal sized 3MM chromatography papers and 2 absorbent pads were soaked in transfer buffer for 10 min and nitrocellulose was soaked in transfer buffer for 5 min. The gel was placed between nitrocellulose and 3MM papers in a proper orientation (ensuring protein transfer to nitrocellulose membrane) and was immersed in transfer buffer. A constant current of 150 mA was applied for 2 hr. The transfer was done with gentle stirring at 4^o C.

Following the transfer, the nitrocellulose membrane was soaked in saline solution with 3% Nonidet P-40 at RT for 30 min (buffer was replaced after 15 min). The membrane was then blocked in saline with 1% BSA and 0.1% Tween 20 for 2 hr at RT. The membrane was incubated with 20 mL 1% BSA in saline + 0.1% Tween 20 containing 2.0 x 10^5 cpm/mL [^{125}I] - IGF-I (20,000 cpm/100 µL) overnight at 4^o C with gentle agitation. The following day, the membrane was washed with saline + 0.1%

Tween 20 for 2 x 15 min at 4^{o} C. This wash was followed by 3 x 15 min washes in saline at 4^{o} C. The membrane was then dried at 37^{o} C for one hr and the membrane was exposed to X-ray film. Autoradiograph was quantified using a Gel Doc system and basic statistics (ex. SD and sample mean) were done using Microsoft Excel. Experimental p values were calculated using the T test and SISA online statistics

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5.0 RESULTS

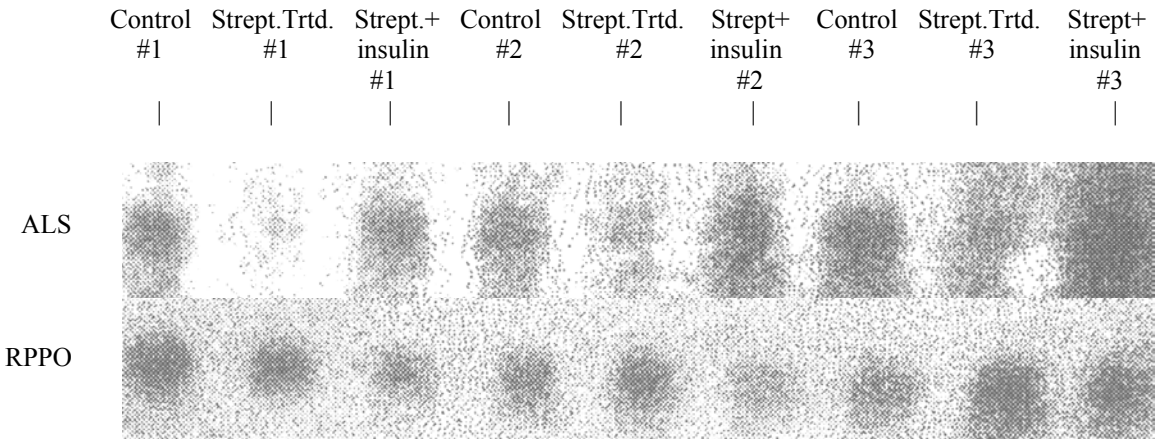
5.1 ALS Gene Expression in Streptozotocin-Induced Diabetic Rat livers

Several studies have demonstrated that insulin induces the expression of ALS (Dai *et al.,* 1994a). It was of interest to investigate whether an *in vivo* change in ALS mRNA levels occurred in conditions which naturally alter insulin levels such as in the case of Type I diabetes. The effect of streptozotocin (STZ)-induced type-I diabetes on ALS mRNA levels were analyzed in the liver tissue of male Wistar rats. Three groups of animals were used for this purpose. One group received citrate buffer injections and served as a negative control. Saline injection was not used a negative control in these studies. A second group was given an i.p. injection of STZ and a third group was given STZ followed by daily insulin injections. All rats were monitored for glucose in urine using Clinistix® (Bayer) glucose strips and were deemed hyperglycemic with an indicated high level of glucose (purple colour on strip). Northern analysis of liver total RNA was performed and data was analyzed for ALS expression. STZ treated rats showed an approximate 50% decrease in ALS gene expression in comparison to the controls (Figure 5.1). The supplement of insulin to STZ-induced diabetic rats brought ALS gene expression to near control levels. All ALS expression was analyzed in comparison to hepatocyte RPPO expression which is used for determination of equal loading amongst samples. In these experiments RPPO expression is used as a measure of base expression in cell samples and is unresponsive to some hormonal regulation such as seen with estradiol (Laborda *et al.,* 1992). The ratio of ALS/RPPO is utilized for the determination of modulator effects on ALS expression.

5.1.1 Discussion

Diabetes has been shown to lead to the development of a number of secondary diseases (Regimbeau *et al.,* 2004, Giovannucci 2001). The importance in determining biochemical changes caused by diabetes may therefore assist in further research to

A.



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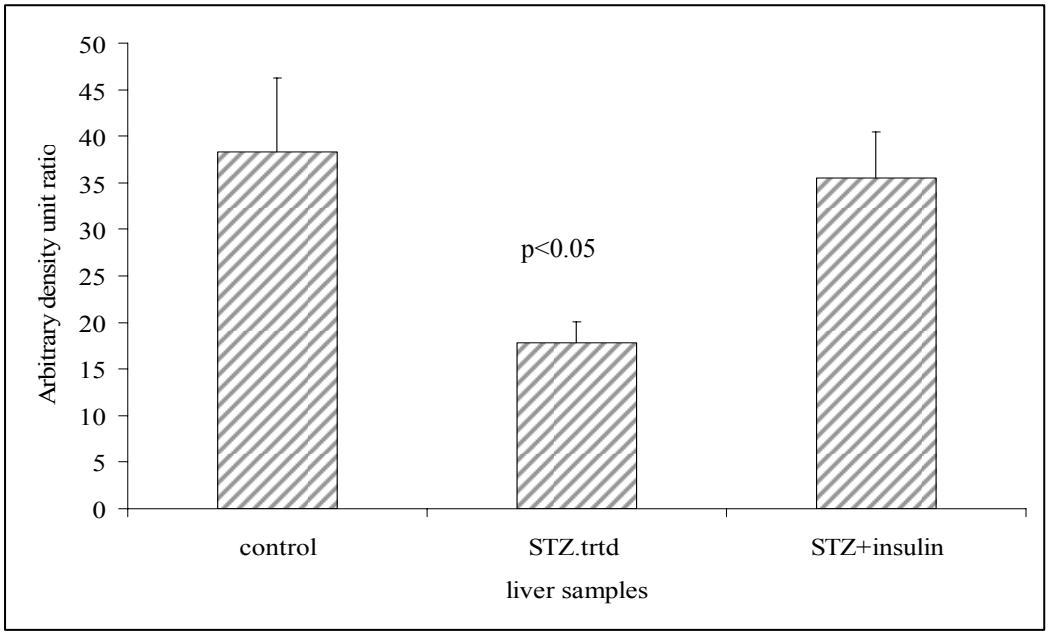


Figure 5.1 Effect of streptozotocin-induced diabetes on ALS gene expression in rat liver tissue. Rat livers were removed following treatments, homogenized in Trizol reagent (Gibco) and stored. RNA was isolated and samples were subjected to Northern analysis. ALS expression was visualized using radiolabelled rALS probe and RPPO expression was analyzed for equivalency of RNA loading. Autoradiographs were produced of x-ray film and blots (A) were analyzed using Gel Documentation system (Bio-Rad) and Quantity One software (Bio-Rad) (B). Values represent the ratio of ALS/RPPO density units. Experiments were performed in triplicate, and means $\pm$ SE is given. T test was performed to determine significance using SISA online statistics. STZ, streptozotocin.

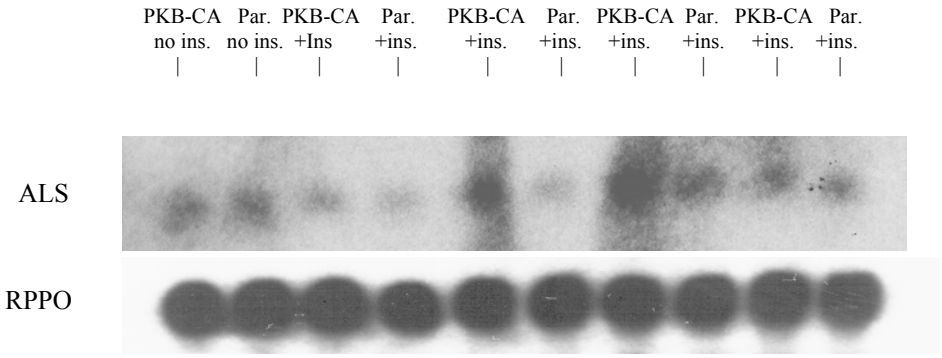
determine how such changes may illicit an effect and cause secondary disease. ALS is found in the bloodstream and acts to regulate the bioavailability of IGF. Regulation of ALS expression and secretion in the liver during Type I Diabetes may be an important factor in downstream effects affecting the availability of IGF-1. Our results indicate that during STZ-induced type 1 diabetes the decrease in insulin secretion results in an approximate 50% decrease in accumulation of ALS mRNA in the rat liver. This large decrease may affect ALS levels in the bloodstream and lead to the possibilities of increased degradation of IGF-1 or increases in IGF-1 activity in target tissues. It has been postulated that IGF-1 activity may be responsible for growth of tumours (Manousos *et al.,* 1999, Khandwala *et al.,* 2000, Macaulay 1991). Diabetes has been correlated with the development of a number of cancer types (Regimbeau *et al.,* 2004, Giovannucci 2001). Decreased formation of ternary complexes in circulation may lead to a loss in IGF-1 control and a resulting IGF-1 mediated stimulation of tumour growth.

5.2 IGFBP-1 Protein Secretion and ALS Gene Expression in PKB Over-expressing HepG2 Cells

5.2.1 ALS Gene Expression

As shown in Figure 5.1 ALS mRNA levels decrease in STZ-induced Type 1 diabetic liver and are restored after insulin supplementation. Decreased and increased insulin may, therefore, have a role in the regulation of ALS gene expression. Protein kinase B (PKB) is an important component of the PI-3 kinase pathway. Insulin is known to activate the PI-3 kinase pathway via signal transduction through the insulin receptor (IR). It is possible that upregulation of ALS mRNA involves upregulation of PKB. The involvement of PKB in the regulation of ALS expression was, therefore, studied through comparison of ALS expression in normal HepG2 and HepG2 cells stably transfected with a constitutively active PKB construct (HepG2-PKB-CA). This experiment was performed in triplicate with and without insulin. Cells were incubated for 12 hr in DMEM (with or without insulin), total RNA was isolated and subjected to Northern analysis. The level of

A.



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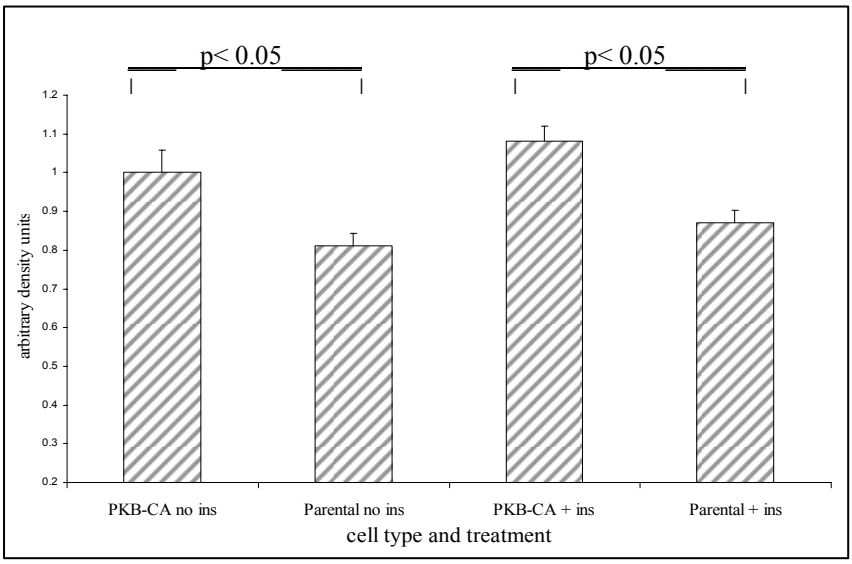


Figure 5.2 The levels of ALS gene expression in parental HepG2 and HepG2-PKB-CA cells. Over-night serum starved cell cultures were incubated for 12 hr at 37^{o} C with or without 10 nM insulin. Total RNA was isolated and changes in ALS mRNA levels were assessed using Northern analysis. ALS expression was visualized using radiolabelled rALS probe and RPPO expression was analyzed for equivalency of RNA loading. Autoradiographs were produced of x-ray film and blots (A) were analyzed using Gel Documentation system (Bio-Rad) and Quantity One software (Bio-Rad). Experiments without insulin were performed in triplicate while those with insulin were performed in quadruplicate. Means $\pm$ S.D. is given (B). T-test was performed separately for those results with insulin and without insulin using SISA online statistics. For both sets comparison of PKB-CA and Parental HepG2 cell ALS expression had a value of $p<0.05$. For comparison between PKB-CA (no ins.) and PKB-CA (ins.): $p<0.5$.

ALS mRNA in parental, non-transfected HepG2 cells was 20% lower than transfected HepG2 cells overexpressing PKB (Figure 5.2). The addition of insulin slightly increased ALS expression in both cell types but increase was insignificant ($p<0.5$). The difference in activation between these two cell types was very similar with and without insulin.

5.2.1.1 Discussion

Our results in HepG2-PKB-CA cells showed a larger ALS expression as compared to parental HepG2 cells with and without insulin signal. Endogenous PKB is activated through the PI-3 kinase pathway which is dependent on PI-3 kinase activation by insulin signal transduction through the IR. The addition of insulin appeared to slightly increase ALS expression in both cell types. Insulin is thought to have no effect on ALS expression in HepG2-PKB-CA cells due to insulin independent activity of the PKB-CA construct. Although statistically there was no change an activity of insulin without the involvement of PKB and possibly the PI-3 kinase pathway may be possible. In later sections, data will be presented showing the effects of PI-3 kinase pathway inhibition on ALS gene expression in H4IIE cells. Nonetheless, increased PKB activity results in larger levels of ALS gene expression in HepG2 PKB-CA cells as compared to parental Hep2 cells. Any difference in ALS gene expression may also be due to clonal effects and without clear quantitative data on PKB levels this result does not prove, without doubt, the involvement of PKB in ALS gene regulation.

Past studies have shown that increased ALS expression does not occur at the transcriptional level in primary hepatocytes in response to insulin. Increased ALS secretion occurs, but occurs without increases in ALS gene expression (Dai *et al.,* 1994a). Our studies in HepG2 hepatoma cells and in STZ-treated rats, however, indicate that ALS mRNA levels may be also affected with changes in insulin and insulin signaling (PKB levels).

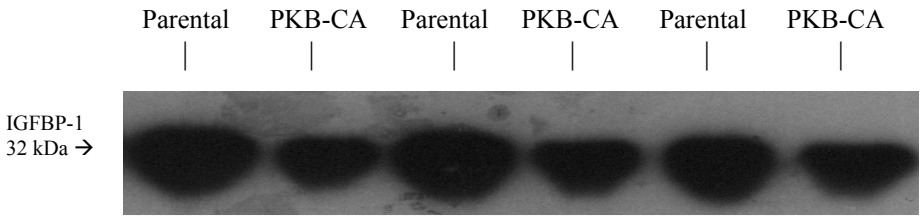
5.2.2 IGFBP-1 Secretion

The repressive effect of insulin on IGFBP-1 expression has been well documented (Suikkari *et al.,* 1989). Some studies have implicated a role for PKB in this activity (Rena *et al.,* 1999, Guo *et al.,* 1999). IGFBP-1 expression was analyzed through Western-ligand blotting. For this, cells were incubated in serum-free media for 6 hr and secreted total protein was isolated from conditioned media. Protein was electrophoresed by non-denaturing SDS-PAGE, transferred to nitrocellulose, probed with [^{125}I]-IGF-1, visualized and protein bands were quantified. IGFBP-1 expression was not investigated in cell lysate due to almost non-existent IGFBP-1. As shown in Figure 5.3, there was approximately 30% less IGFBP-1 secretion in HepG2-PKB-CA cells into media as compared to parental HepG2 cells. Insulin was not added in these experiments due to the supposed lack of importance with an already well characterized regulatory pattern through the PI-3 kinase pathway.

5.2.2.1 Discussion

IGFBP-1 expression and secretion has been shown to be negatively regulated by insulin (Suikkari *et al.,* 1989). It has been suggested that PKB may have a role in signaling for the suppression of IGFBP-1 expression. This signaling may occur through mTOR, p70^{s6k}, and FKHR related protein which is thought to bind to the IRE on the IGFBP-1 gene promoter region and inhibit IGFBP-1 expression. FKHR is activated through phosphorylation by PKB (Rena *et al.,* 1999, Patel *et al.,* 2002). With increased PKB activity in HepG2-PKB-CA cells, there was an associated IGFBP-1 secretion which was 30% lower than Parental HepG2 cells (Figure 5.3). Thus it appears that IGFBP-1 secretion may be partially regulated by active PKB. This data may agree with qualitative observations made by Rena *et al.* (1999) and Patel *et al.* (2002), but the involvement of FKHR related protein in gene regulation was not investigated. Any difference in IGFBP-1 secretion may be due to clonal effects and without clear quantitative data on PKB levels this result does not prove the involvement of PKB in the negative regulation of IGFBP-1 by insulin.

A.



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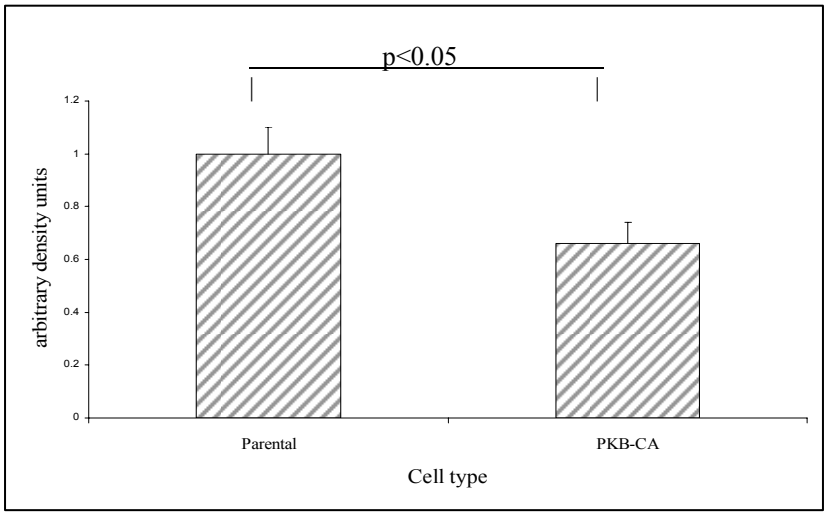

Figure 5.3 The levels of IGFBP-1 secretion in parental HepG2 and HepG2-PKB-CA cells. Over-night serum starved cells were incubated for 6 hr at 37^{o} C. Following incubation, media was stored at -80^{o} C after addition of protease inhibitors. The thawed samples were subjected to desalting on a Sephadex G-50 column, protein fractions were collected and lyophilized. Following lyophilization, samples were resuspended in sample buffer and 50 ug was loaded onto a 12 % SDS PAGE gel and then transferred to nitrocellulose. Blots were probed with radiolabelled IGF-1 and autoradiographs analyzed. Blots shown above represent averages of three independent sets of data analyzed (A). Autoradiographs were analyzed using densitometry with the Gel Documentation System (Bio-Rad) and Quantity One software program (Bio-Rad) (B). Means $\pm$ S.D. are given as well as T test values.

Regulation of IGFBP-1 levels in circulation through insulin signaling reveals a secondary mechanism of hypoglycemic potential in hepatic cells. In states of increased insulin levels IGFBP-1 is repressed (Suikkari *et al.,* 1989a and b, Holly *et al.,* 1988). Our results qualitatively may indicate that this repression is controlled through insulin mediated activation of PKB and the PI-3 kinase pathway. Repression of IGFBP-1 expression may result in a larger available pool of free IGF-1 acting in an accumulative manner with insulin to bring down levels of glucose in circulation. This IGF-I mediated event may occur as a long term effect compared to the short term effects of insulin. Although this is a possibility it may also be possible that increased free IGF-1 will bind in ternary complexes and increase the half life of IGF-I.

5.3 Secretion of IGFBP-1 Protein in H4IIE Cells

5.3.1 Effects of Incubation Time on IGFBP-1 Protein Secretion

H4IIE hepatoma cells have been shown to express and secrete IGFBP-1 (Orlowski *et al.,* 1990, Unterman *et al.,* 1991) and therefore provide a suitable model for protein expression analysis. The effect of incubation time on IGFBP-1 expression in H4IIE cells was first investigated in order to determine optimum time for expressional analysis in experiments determining the transcriptional effects of cAMP, glucose, dexamethasone, insulin, and GH. As IGFBP-1 is a secreted protein, all proteins were analyzed in conditioned media. The effects of various modulators on IGFBP-1 protein expression as a function of incubation time are shown in Figure 5.4a (blots), Figure 5.4b (quantitation bars) and Figure 5.5. A control H4IIE time course was done for comparison to those with metabolic modulators. Blots were analyzed using measurements of optical density and a 5.0-fold increase in protein expression over a 36 hr period was observed in control samples (Figure 5.4a, A; Figure 5.4b, A, Figure 5.5). Again, using optical density measurement, increases similar to control were measured with cAMP, glucose and dexamethasone incubation. These modulators showed relatively no difference in effect as compared to control samples (Figure 5.4a and Figure 5.4b; B, C and D). The effect of insulin over a 36 hr period showed a steady increase in IGFBP-1 expression. The

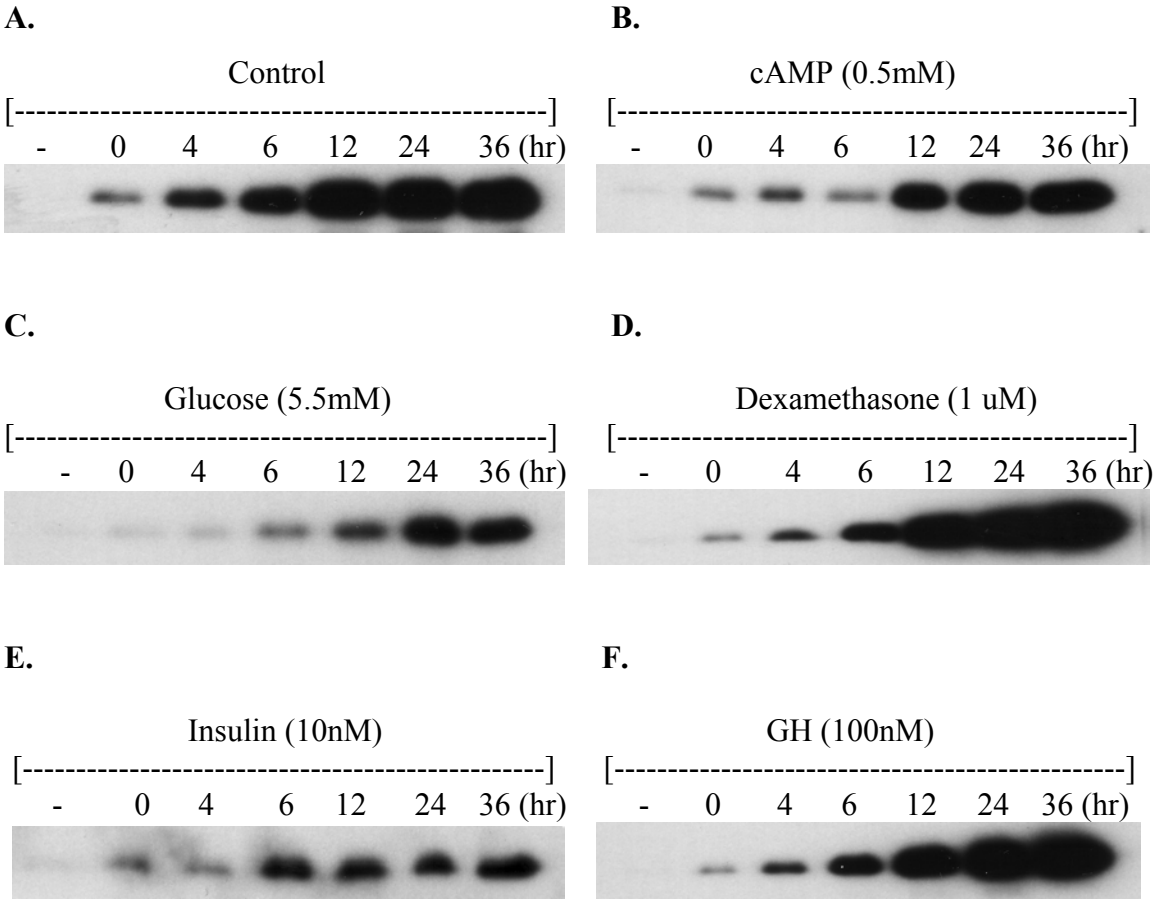

Figure 5.4a The level of IGFBP-1 secretion in response to modulator time of incubation in H4IIE cells. Cell cultures were incubated for 0 to 36 hr with or without modulators. The experiments are shown above for treatments with (A) Control, (B) cAMP(0.5 mM), (C) Glucose (5.5 mM), (D) Dexamethasone (1 μM), (E) Insulin (10 nM), (F) GH (100 nM). Media was stored at -80^{o} C after adding protease inhibitors. The thawed samples were run through Sephadex G-50 column and protein fractions collected and lyophilized. The samples were resolubilized in 50 mM Tris-HCl (pH 7.4). Western Ligand analysis was performed on these samples and autoradiographs produced were analyzed. The quantitative data is shown in Figure 5.4b. GH, Growth Hormone.

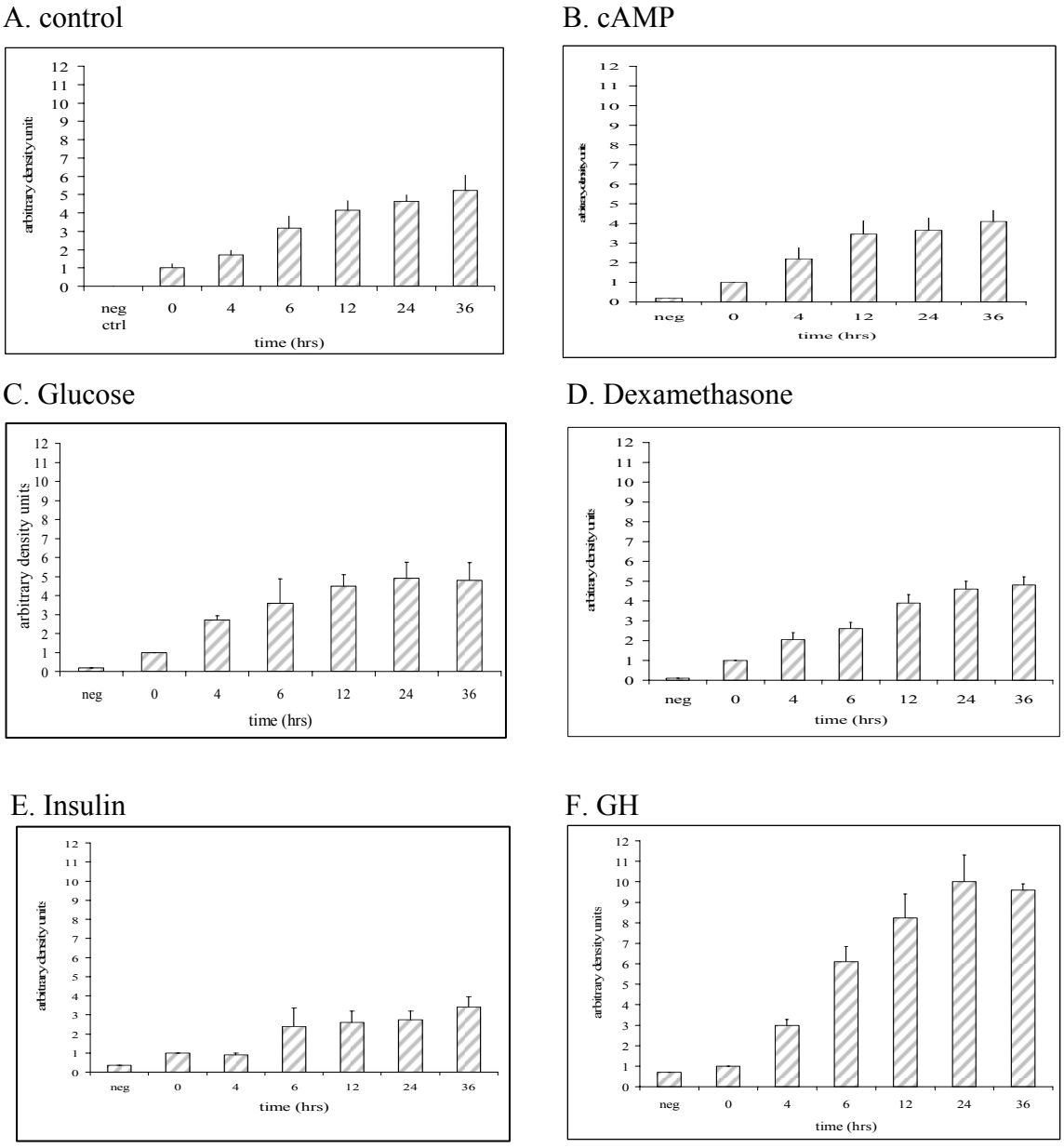

Figure 5.4b The level of IGFBP-1 secretion in response to modulator time of incubation in H4IIE cells. Autoradiographs were analyzed using densitometry with the Gel Documentation System (Bio-Rad) and Quantity One software program (Bio-Rad). The experiments are shown above for treatments with (A) Control, (B) cAMP(0.5 mM), (C) Glucose (5.5 mM), (D) Dexamethasone (1 μM), (E) Insulin (10 nM), (F) GH (100 nM). Values presented are the averages of two duplicated independent experiments (4 values). S.E. of mean are shown as vertical bars in respective sample plots. $p<0.5$ for all values obtained less than 6 hr and $p<0.05$ for values between 6 and 12 hr. For values obtained at 24 and 36 hr $p< 0.005$ except for insulin samples where these values are $p<0.05$.

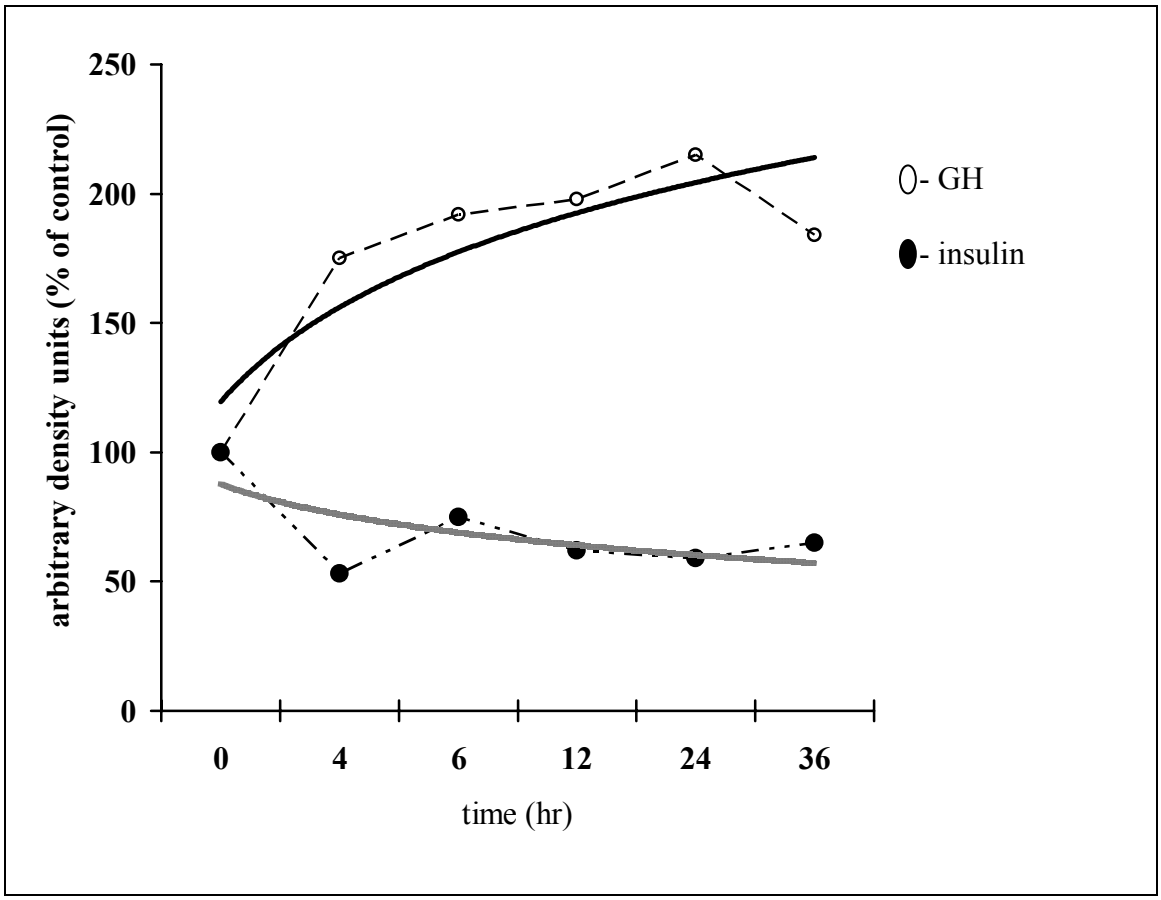

Figure 5.5. H4IIE cell IGFBP-1 secretion in response to incubation time with insulin and GH effect represented as percentage of control. In this plot the arbitrary density units of the control time-dependent expression (Figure 5.4b, A) were given a value of 100% in each timed sample. The corresponding values obtained with GH and insulin (Figure 5.4b, E and F) were calculated with respect to the control values. The resulting curves correspond to the deviation of H4IIE IGFBP-1 expression from control due to the presence of metabolic modulator. For the statistics on individual samples (S.E., p-value) refer to Figure 5.4b. Trendlines (log derived) of both insulin and GH effects were added.

maximum expression was only 3.0-fold above the insulin zero time control and the maximum value for insulin was 40% lower than the maximum value obtained in control samples (Figure 5.4a and Figure 5.4b, E; Figure 5.5). In contrast, the IGFBP-1 protein secretion in GH treated H4IIE cells was 2-fold greater than the maximal response seen in control samples (Figure 5.4a and Figure 5.4b, F, Figure 5.5). A 10-fold increase in H4IIE IGFBP-1 levels were observed with GH after 24 hr with comparison to the zero time GH control.

5.3.2 The Effects of Varying Concentrations of Modulators on IGFBP-1 Protein Secretion

Next the effects of varying concentrations of metabolic modulators on IGFBP-1 secretion were analyzed in H4IIE cells. For this, H4IIE cells were incubated 6 hr (as determined in time course) in the absence and presence of modulators added to serum-free media. Total protein was isolated from conditioned media and subjected to Western ligand analysis as described in material and methods section. The results are shown in Figure 5.6a (blots) and Figure 5.6b (quantitation bars). cAMP and dexamethasone had no effect on expression as compared to controls (Figure 5.6a and Figure 5.6b; A and B). GH and glucose treatments increased IGFBP-1 levels 4.0-fold and 2.0 fold, respectively, as compared to controls. The effect of GH was seen as a plateau, with little change with increasing concentrations, whereas the change with glucose was linear (Figure 5.6a and Figure 5.6b; C and D). Insulin treatment of H4IIE cells resulted in a linear decrease in IGFBP-1 expression. The largest decrease was observed with 100 nM insulin, decreasing IGFBP-1 expression to 50% of the control (Figure 5.6a and Figure 5.6b, E).

5.3.3 The Involvement of PI-3 kinase and MAP Kinase Pathway Inhibitors on the Regulation of IGFBP-1 by Metabolic Modulators

Wortmannin and PD98059 are inhibitors of the PI-3 kinase and MAP kinase pathways, respectively (Lam *et al*., 1994, Alessi *et al*., 1995). Experiments were

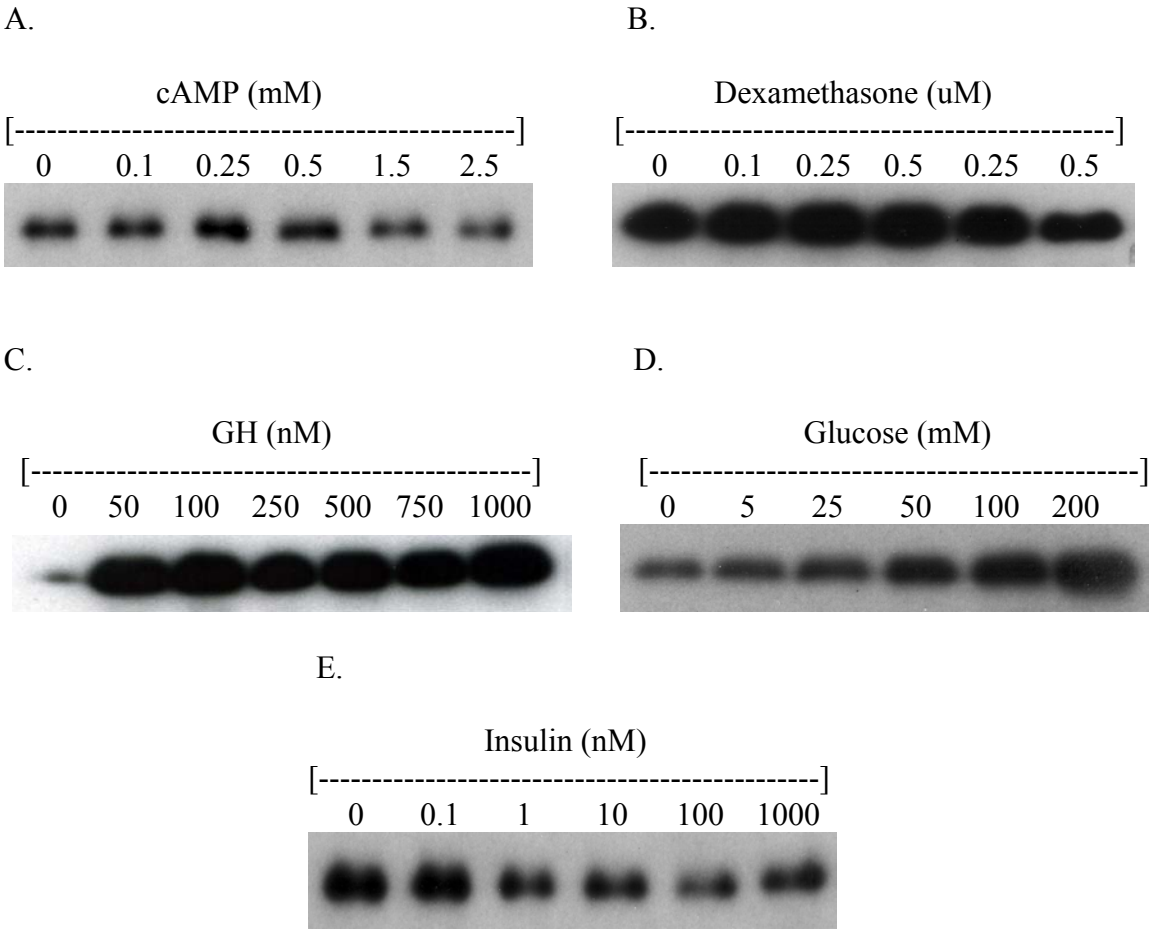

Figure 5.6a Effects of varying modulator concentration on IGFBP-1 secretion in H4IIE cells. Cells were treated with varying doses of modulators in serum-free media for 6 hr. The experiments are shown above for treatments with (A) cAMP, (B) Dexamethasone, (C) GH , (D) Glucose and (E) Insulin. Conditioned media was removed after treatment and protease inhibitor cocktail was added (10ul/10mL media) and stored at -80^{o} C. Samples were subjected to desalting on a Sephadex G-50 column and protein fractions were collected and lyophilized. Following lyophilization, samples were resuspended in 50 mM Tris buffer and concentrated 80 to 100x. 50ug of the sample was loaded onto a 12 % SDS PAGE gel (low denaturing) and transferred to nitrocellulose membrane. Blots were probed with radiolabelled IGF-1 and autoradiographs analyzed. Exposure times varied. Each experiment was done in duplicate and repeated independantly two times (4 values).

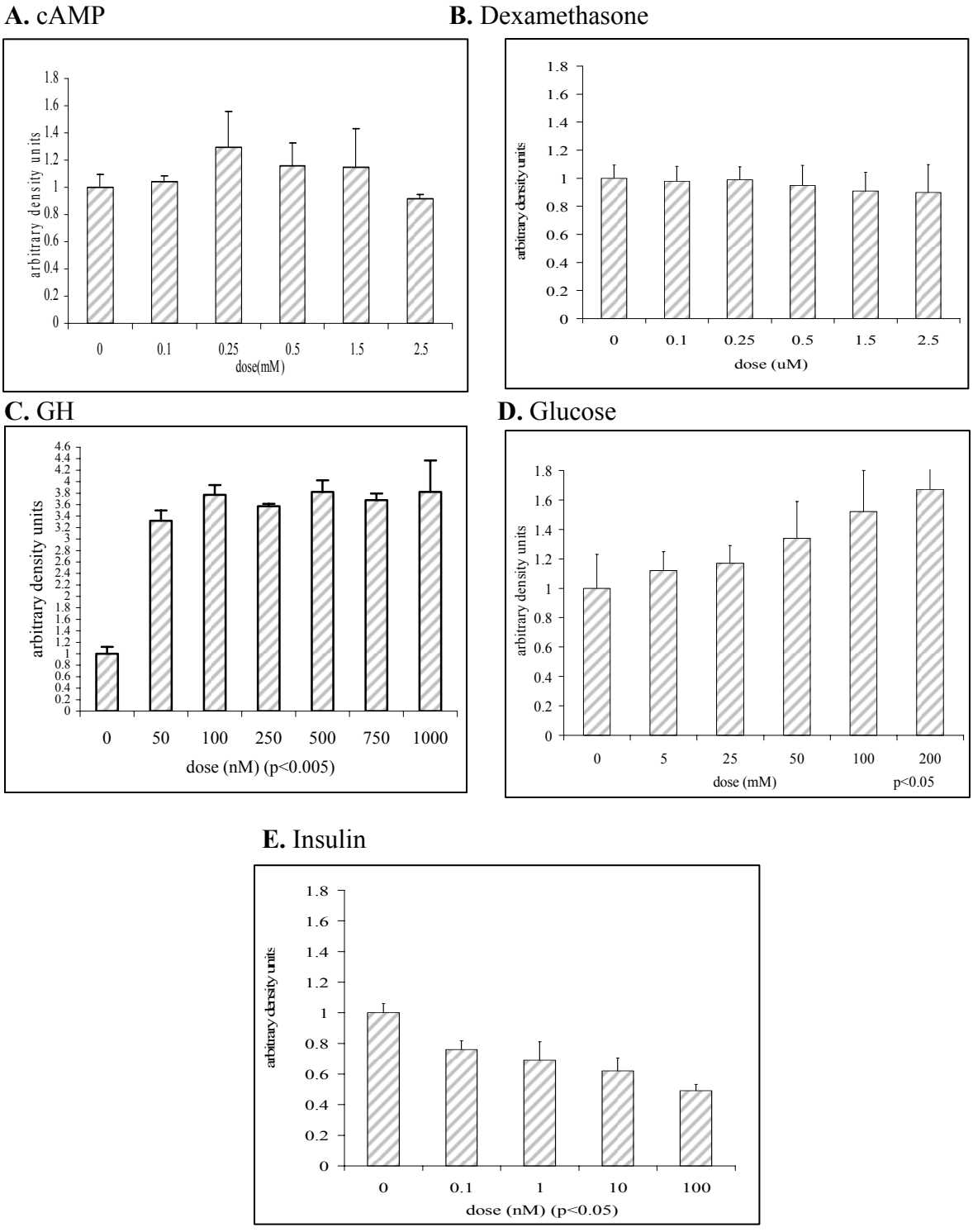

Figure 5.6b Effects of varying modulator concentration on IGFBP-1 secretion in H4IIE cells. Autoradiographs were analyzed using densitometry with the Gel Documentation System (Bio-Rad) and Quantity One software program (Bio-Rad) as well as Scion imaging software. The experiments are shown above for treatments with (A) cAMP, (B) Dexamethasone, (C) GH , (D) Glucose and (E) Insulin. Values presented are the averages of two independent experiments done in duplicate (4 values). S.E. is given as an error bar in respective sample plot. Significant P values are given in x axis title and represent the comparison between individual samples and the zero time control.

performed to investigate the effects of wortmannin and PD98059 on the ability of insulin, GH and glucose to affect IGFBP-1 secretion. Dexamethasone and cAMP previously did not show an affect on IGFBP-1 secretion and were therefore excluded.

In all experiments, cells were pre-incubated for 30 min with wortmannin or PD98059 followed by 6 hr incubations with various modulators and/or inhibitors. It was previously determined that 6 hr incubation was adequate in order to see effects of metabolic modulators (Figure 5.4b). Conditioned media was collected and protein was partially purified as described previously. Protein was subjected to Western ligand analysis and blots analyzed for relative IGFBP-1 secretion. The results are shown in Figure 5.7 (blots and quantitation bars).

The effects of metabolic modulators as well as wortmannin and PD98059 were assessed for their effects on IGFBP-1 secretion. GH and glucose caused 3-fold and 2-fold increases in IGFBP-1, respectively (Figure 5.7, A and B). Insulin appeared to decrease IGFBP-1 secretion by about 25% as compared to control, but this decrease was statistically insignificant (Figure 5.7, C). All effects of modulators tested were comparable to results obtained in Figures 5.5 and 5.6. The pre-incubation of cells with wortmannin alone resulted in an average increase of 2.0-fold as compared to the control (Figure 5.7). The pre-incubation of cells with PD98059 alone decreased IGFBP-1 expression to an average value of 50% of control (Figure 5.7)

Examination of the results indicated that wortmannin did not affect the regulation of IGFBP-1 secretion by GH (Figure 5.7, A). The effect of wortmannin on glucose mediated upregulation of IGFBP-1 appeared to be additive resulting in a 1.5-fold increase over glucose and wortmannin controls, but this was not supported by the statistics and therefore is not statistically significant (Figure 5.7, B). There was, however, a significant 50% decrease in IGFBP-1 levels with the addition of insulin to wortmannin as compared to the wortmannin control which was also 1.5-fold higher than the insulin control.

The effects of PD98059 on all modulator effects showed significant increases as compared to the PD98059 control. Increases in insulin, glucose and GH were 2-fold, 4-fold and 4-fold, respectively. GH and glucose mediated regulation of IGFBP-1 levels, in the presence of PD98059, increased to levels comparable with respective modulator controls. The effect of PD98059 on insulin mediated effects was additive with a 1.4-fold

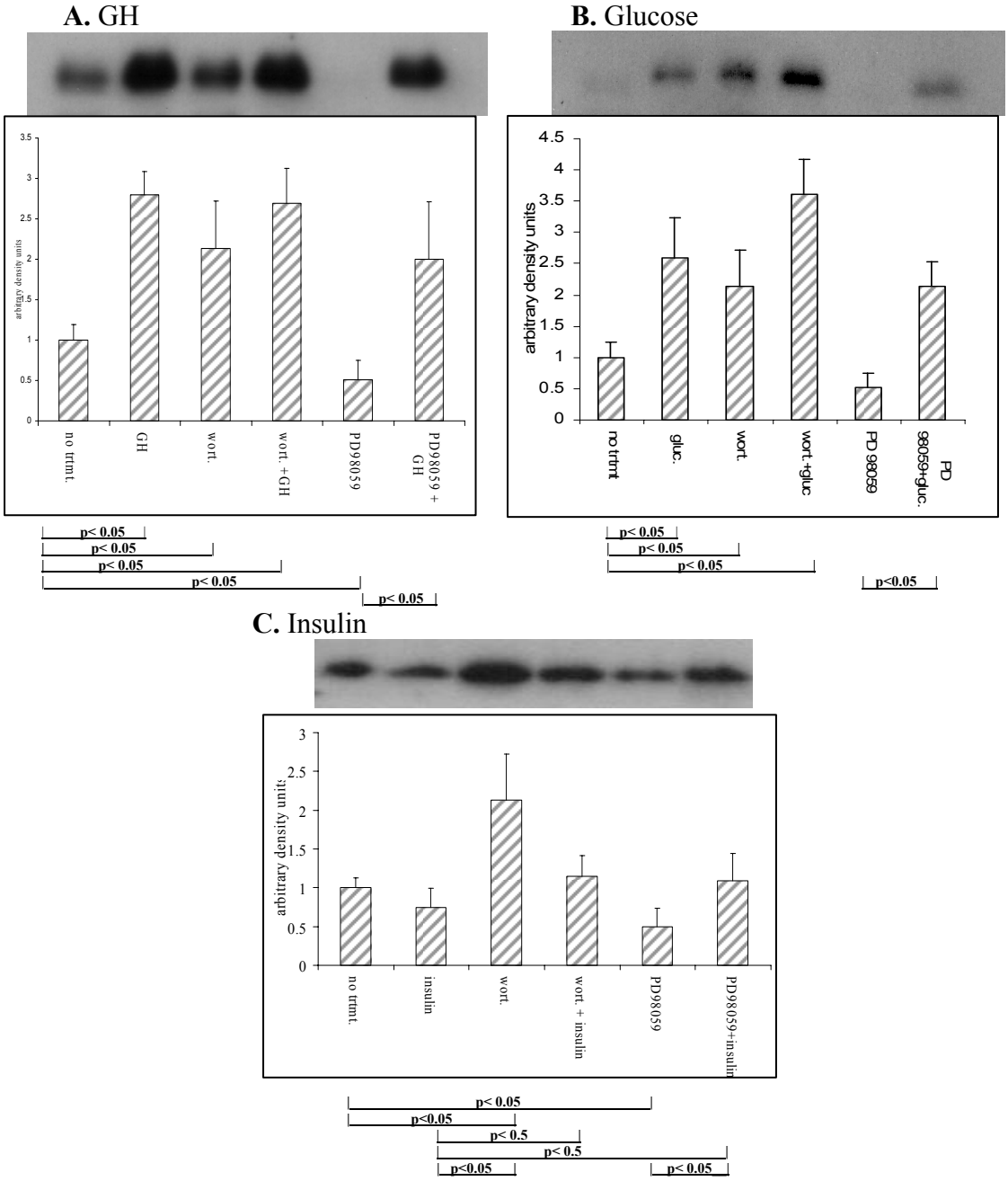

Figure 5.7 The effects of wortmannin and PD98059 on GH, glucose and insulin regulated IGFBP-1 secretion. (0.2 uM wortmannin, 10 uM PD98059, 100 nM GH, 22 mM glucose, 10 nM insulin). H4IIE cell cultures were pre-treated with inhibitors for 30 min, followed by the addition of metabolic modulators (except for controls) and incubated for 6 hr. Blots were probed with radiolabelled IGF-1 and autoradiographs analyzed. The experiments are shown above for treatments with (A) GH, (B) glucose and (C) Insulin. Blots shown above represent one of two sets of data analyzed for H4IIE IGFBP-1 expression and secretion into conditioned media. Autoradiographs were analyzed using densitometry with the Gel Documentation System (Bio-Rad) and Quantity One software program (Bio-Rad). Values presented are the averages of two duplicated independent experiments (4 values). Significant P values are indicated below Figures according to the pairs of samples tested by the T test.

increase in IGFBP-1 levels as compared to the insulin control. However, this assumption is not supported by statistics in this case.

5.3.4 Discussion

GH has previously been shown to either suppress or not effect IGFBP-1 secretion in different cell types (Seneviratne *et al.,* 1990; Orlowski *et al.,* 1991). Our results indicate that GH did strongly affect IGFBP-1 secretion over time (Figure 5.4b, Figure 5.5). Also, in dose dependency studies, the results indicated a maximal 4-fold increase in IGFBP-1 secretion (Figure 5.6b). There was no change in IGFBP-1 secretion as GH concentrations increased. This may indicate that changes in IGFBP-1 secretion due to GH concentrations tested were above a threshold to see dose dependent increases in the H4IIE cell response. This was also comparable to results where 6 hr incubation with a 100 nM GH dosage increased IGFBP-1 concentrations 3-fold above untreated samples (Figure 5.7). The effect of PI-3 kinase and MAP kinase pathway inhibition through wortmannin and PD98059 treatments, respectively, and the positive effect of GH on IGFBP-1 protein secretion showed limited involvement of PI-3 and MAP kinase pathways in GH mediated effects.

PD98059 treatment, alone, brought IGFBP-1 secretion to levels below control values. This may indicate a possible importance of the MAP kinase pathway in the constitutive secretion of IGFBP-1 and more specifically the possible control of IGFBP-1 transcription through Erk1 (Band *et al.,* 1997). Wortmannin treatment increased IGFBP-1 levels 1.25-fold, suggesting a possible inhibitory role for the PI-3 kinase pathway. These were common characteristics in all inhibitor studies completed using wortmannin and PD98059.

Although not much is known about the effects of glucose on IGFBP-1 secretion, Snyder *et al.,* (1990) have suggested that glucose uptake may have a role in the regulation of IGFBP-1 secretion. A positive glucose regulation of IGFBP-1 would result in decreased availability of IGF in circulation, but at the same time, increase IGF half life and have possible long-term effects on glucose control in circulation. Our data suggests that glucose positively regulates IGFBP-1 secretion (Figure 5.6b, D; Figure 5.4b, C). A

similar 5-fold maximal IGFBP-1 secretion is seen in both glucose and control secretion profiles (Figure 5.4b). The initial rate of increase was higher with glucose than in control samples (e.g. compare 4 hr time points). Similarly, the maximum increase with glucose was observed at 24 hr as compared to control secretion that was reached at 36 hr. To further support the positive effect of glucose on IGFBP-1 secretion, dose dependency also showed a maximum 2-fold increase in IGFBP-1 secretion with increasing glucose concentration (Figure 5.6b). There was no effect of PD98059 on glucose-mediated secretion of IGFBP-1. However, wortmannin had an additive effect on glucose mediated upreglation of IGFBP-1 indicating a potential involvement of the PI-3 kinase pathway. Although the exact mechanism by which glucose mediates IGFBP-1 upregulation is unknown, it is probable that the observed positive regulation of IGFBP-1 by glucose may be the result of increased cell growth due to increased glucose utilization by H4IIE cells.

Other studies in H4IIE cells have shown that cAMP inhibits IGFBP-1 secretion (Unterman *et al.,* 1991). The completed time course studies show a slight repressive effect with IGFBP-1 secretion increasing to 4-fold as compared to the control secretion increase to a maximum of 5-fold (Figure 5.4b, B). However, cAMP repression was not clearly shown in dose dependent studies (Figure 5.6b, A). According to these results repression of IGFBP-1 secretion through cAMP signal is minimal and insignificant within H4IIE cells (Figure 5.6b, A).

The ability of insulin to repress IGFBP-1 activity has been well documented in H4IIE cells (Orlowski *et al.,* 1990) as well as in *in vivo* studies (Suikkari *et al.,* 1989). Our results support these data as both insulin time course and dose dependency experiments exhibited a decrease in IGFBP-1 secretion (Figure 5.4b and Figure 5.6b). The maximum inhibitory effect was observed with 100nM insulin.

Examination of the effects of wortmannin and PD98059 treatment on the repressive effect of insulin on IGFBP-1 secretion showed involvement of the PI-3 kinase and MAP kinase pathways. In the presence of wortmannin, insulin repression may be reversed to values above those of the insulin control. This data is inconclusive as $p<0.5$, representing a statistical insignificance. However, the ability of insulin to repress IGFBP-1 secretion may be inhibited when the PI-3 kinase pathway is blocked as compared to the insulin control. Future tests must be conducted to confirm. This idea may be supported by

data obtained with HepG2-PKB-CA that showed increased repression of IGFBP-1 as compared to Normal HepG2 with and without insulin treatment. The level of IGFBP-1 secretion with insulin and wortmannin was significantly lower than wortmannin treatment alone. This is suggestive of an activity of the PI-3 kinase pathway regardless of the insulin signal or an independent function of the inhibitor. In contrast, when insulin was added to PD98059 inhibited H4IIE, the repression of secretion was totally reversed resulting in a large increase in IGFBP-1 secretion as compared to PD98059 controls. Thus a product of the activated insulin signaling cascade may be interacting with components of the inhibited MAP kinase pathway and allowing inhibition to be reversed. Interplay between these two pathways has been suggested and may prove to be an important property in IGFBP-1 regulation (Band *et al.,* 1997; Patel *et al.,* 2002) (Figure 5.7b).

All of our data indicated no significant effect of dexamethasone on IGFBP-1 secretion in H4IIE cells (Figure 5.4b and Figure 5.6b) This is in contrast to a previous report in which an increase of IGFBP-1 mRNA by 10-fold was reported (Orlowski *et al.,* 1990). They, however, did not measure secreted IGFBP-1 levels. IGFBP-1 secretion, although related to expression, may occur as a result of separate cellular mechanisms.

5.4 ALS Gene Expression in H4IIE Cells

5.4.1 Effect of Incubation Time on ALS Gene Expression

The effect of metabolic modulators on ALS gene expression as a function of incubation time was examined in H4IIE cells. In these studies, H4IIE cells were chosen due to their known sensitivity to the metabolic modulators and their marked similarity to the differentiated hepatocyte. It is known that hepatocytes are the primary site of ALS expression and as such H4IIE cells provide a suitable cell model for analysis. In addition, the experiments were performed to determine the effectiveness of the rALS and RPPO cDNA probes used in Northern analysis. A time course control was not performed in this

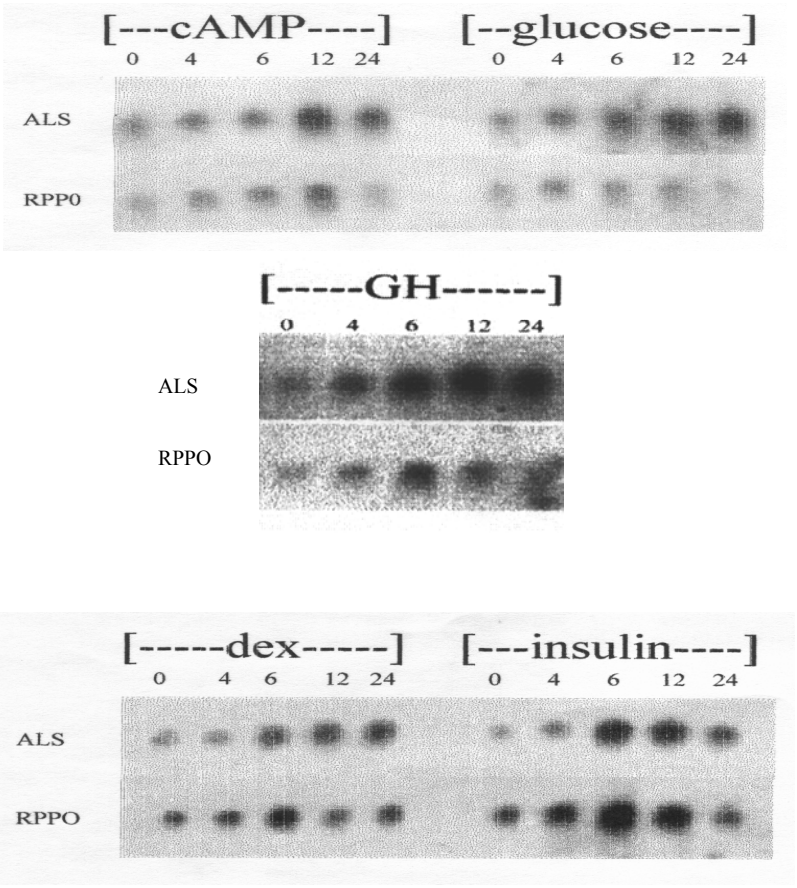

Figure 5.8a. The effect of modulators on ALS gene expression in response to H4IIE cell incubation time. Changes in ALS gene expression were analyzed by Northern analysis in comparison to RPPO expression. RPPO mRNA expression is an accepted measure of equivalency amongst loaded RNA samples. Due to apparent unequal loading the data is represented as a ratio of ALS:RPPO. All controls (zero time) were arbitrarily set to equal "1" arbitrary density unit ratio. Changes in ALS expression were a reflection of changes in this ratio. The concentrations of metabolic modulators were: GH (100 nM); cAMP (0.5 mM); glucose (5.5 mM); dexamethasone (1 uM); and insulin (10 nM). The cells were harvested at 0, 4, 6, 12 and 24 hr after initiation of the experiment and mRNA was isolated and analyzed by Northern analysis.

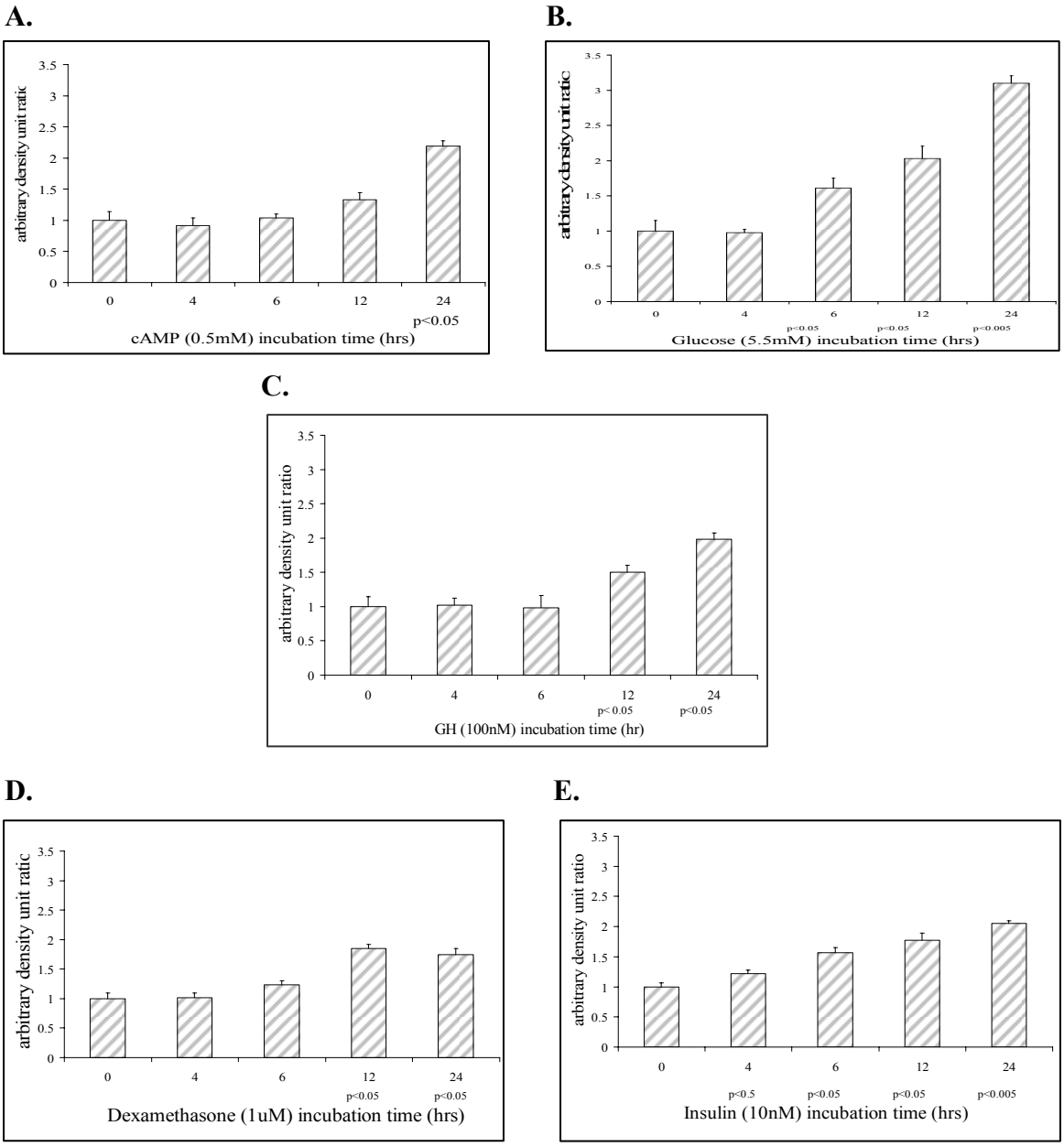

Figure 5.8b. The effect of modulators on ALS gene expression in response to H4IIE cell incubation time. Changes in ALS mRNA levels were assessed using Northern analysis and RPPO expression was analyzed for equivalency of RNA loading. Autoradiographs (Figure 5.8a) were analyzed for band intensity using Gel Doc System and Quantity One software (Bio-Rad). To account for differences in loading the arbitrary density units of ALS were divided by those of RPPO and represented as a ratio. Expression at time zero was given a value of one arbitrary density unit ratio. Experiments were performed using cAMP (A), glucose (B), GH (C), dexamethasone (D) and insulin (E). Values presented are the averages of two independent experiments +/- S.D. Significant P values are indicated in the Figures compared to control.

experiment due to the eventual deterioration of the rALS probe. The metabolic modulators chosen for this study were insulin, cAMP, glucose, dexamethasone and GH. The effects of various modulators on ALS gene expression as a function of incubation time are shown in Figure 5.8a (blots) and Figure 5.8b (quantitation bars). Dexamethasone, cAMP, GH and glucose increased ALS gene expression 2-fold, 2-fold, 2-fold and 3-fold, respectively, over a period of 24 hr with a lag period of 4 to 6 hr (Figure 5.8b, D, A, C and B). In the presence of insulin, ALS mRNA accumulated immediately and increased to a maximum of 2-fold in 24 hr (Figure 5.8b, E).

5.4.2 Effects of Varying Concentrations of Modulators on ALS Gene Expression

Accumulation of ALS mRNA as a function of modulator concentration was determined in H4IIE cells. From the time course experiments, 12 hr incubation time was used in these experiments. Metabolic modulators were added to serum-free media and incubated for 12 hr in a 37^{o} C CO_2 incubator. The effects of various modulators on ALS gene expression as a function of varied concentrations of metabolic modulators are shown in Figure 5.9a (blots) and Figure 5.9b (quantitation bars). The effect of cAMP on ALS mRNA accumulation was negative overall with increases in cAMP concentrations (0.5 to 10 mM) resulting in a maximal decrease in ALS mRNA levels to 64% of the control value (Figure 5.9b, A). The effect of glucose and dexamethasone on ALS gene expression was positive with maximal 3.0 and 2.5-fold increases, respectively (Figure 5.9b, B and D). The stimulation of ALS expression by dexamethasone plateaued at a 0.5 uM dexamethasone concentration (Figure 5.9b, E). Effects of increasing GH concentrations were not evident at lower concentrations tested (<100nM). At concentrations higher than 100 nM, ALS gene expression dramatically increased 4-fold at a GH concentration of 500 nM (Figure 5.9b, C). In these studies, with measurement of the ALS/RPPO OD ratio, insulin showed a 2-fold increase in ALS mRNA with a maximal expression at 10-100 nM (Figure 5.9b; E). It was noted in these experiments that 12 hr H4IIE incubations with cAMP (0.5 mM), glucose (5.5 mM) and GH (100nM) differed somewhat from the expression seen at like concentrations and times of incubation in time course studies (Figure 5.8b). In these cases increases seen in time

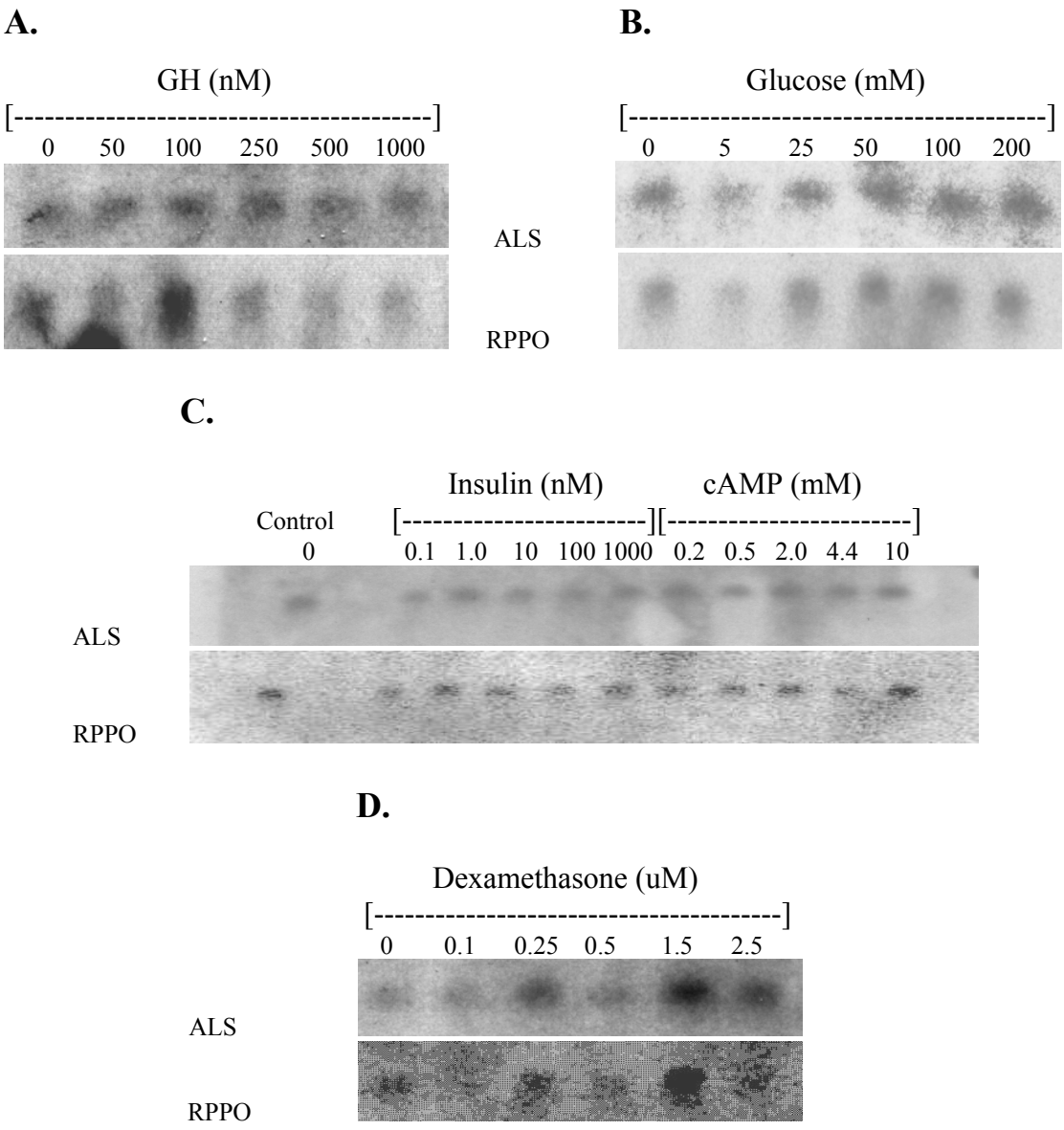

Figure 5.9a Effects of varying modulator concentrations on ALS gene expression in H4IIE cells Changes in ALS gene expression were analyzed in comparison to RPPO gene expression for assurance of equivalency amongst samples. The metabolic modulators with which the H4IIE cells were treated and the concentrations used were as shown above: GH (0-1000nM) (A); glucose (0-200mM) (B); insulin (0-1000nM) and cAMP (0-10mM) (C); dexamethasone (0-2.5 uM) (D). Autoradiographs were produced on X-OMAT 5 Kodak film and/or done on phosphoimager (Bio-Rad).

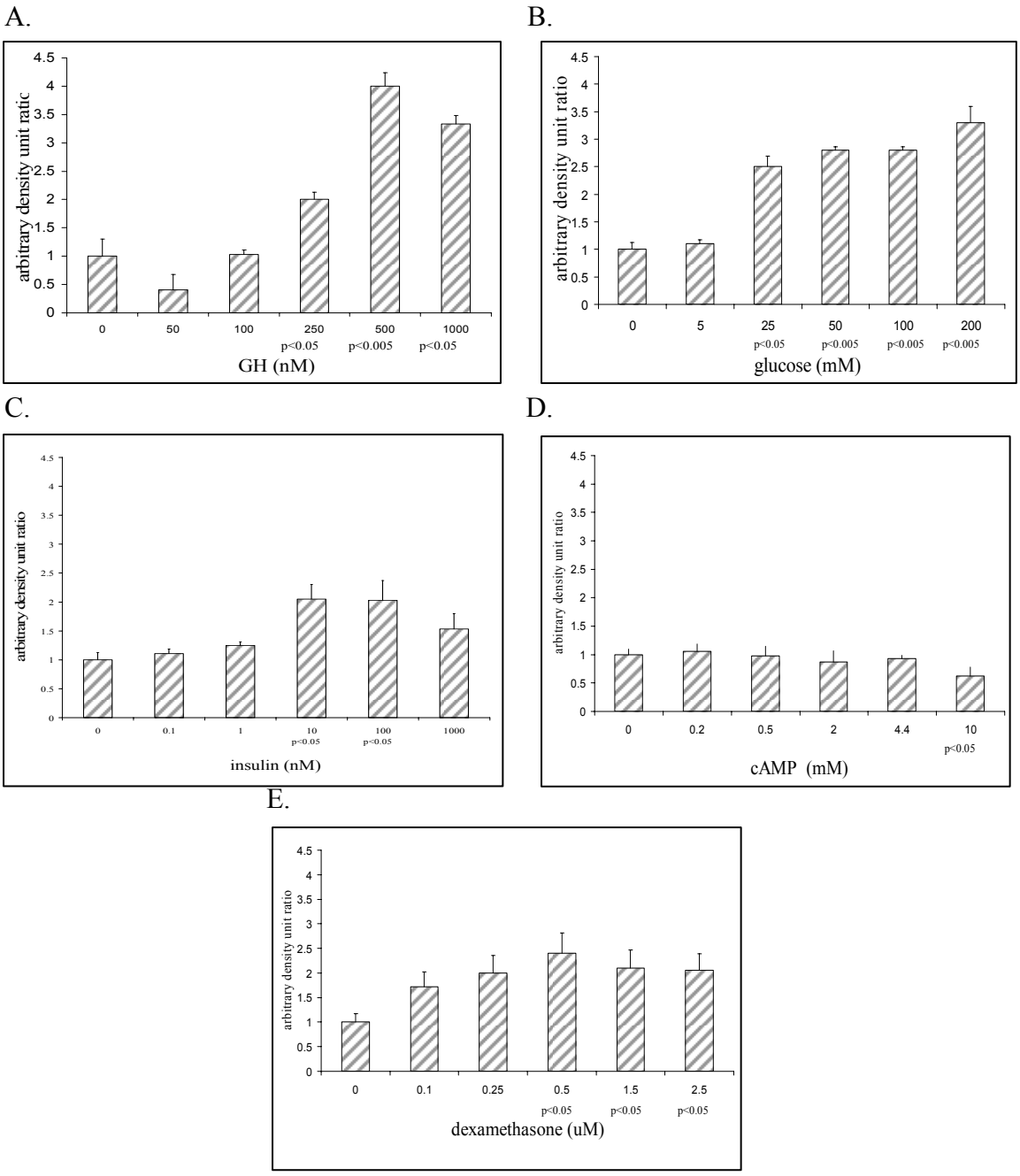

Figure 5.9b Effects of varying modulator concentrations on ALS gene expression in H4IIE cells Changes in ALS mRNA levels were assessed using Northern analysis. Experiments are shown as: GH (0-1000nM) (A); glucose (0-200mM) (B); insulin (0-1000nM) and cAMP (0-10mM) (C); dexamethasone (0-2.5 uM) (D). RPPO expression was analyzed for equivalency of RNA loading. Autoradiographs were analyzed for band intensity using Gel Doc System and Quantity One software (Bio-Rad). All expression at time zero was given a value of one arbitrary density unit. To account for differences in loading the arbitrary density units of ALS were divided by those of RPPO and represented as a ratio above. All ratios of expression at time zero were given a value of one arbitrary density unit + S.D.. Values are the averages of two independent experiments. P values are indicated in the figures.

course studies were not observed. Small differences should be expected amongst separate experiments. However additional error may have resulted due to probes, hormonal or mRNA degradation over time.

5.4.3 Effects of cAMP and Insulin Alone or in Combination on ALS Gene Expression in H4IIE Cells

This study was done to investigate the effects of insulin and cAMP, alone or in combination, on ALS gene expression in H4IIE cells. The results are shown in Figure 5.10 (blots and quantitation bars). In this experiment statistical analysis using the T test gave p values < 0.5 which deems all this data to be statistically insignificant. It will, however, still be presented. A 6 hr cAMP treatment of H4IIE cells showed a 20% decrease in ALS mRNA versus control while insulin treatment for the same time period showed an insignificant 5% increase in ALS mRNA accumulation. These results, in comparison with cAMP and insulin time dependent effects (Figure 5.8b) with like concentrations of insulin and cAMP show similar results. However, the insulin effect at 6 hr incubation was a 1.5- fold increase and cAMP showed a small insignificant change at 6 hr (Figure 5.8b). With both cAMP and insulin added for six hr ALS gene expression decreased 26% compared to non-treated control. This 26% decrease in ALS was similar to the effects of cAMP alone. Insulin treatment for 12 hr displayed a 15% increase in ALS mRNA which is a small increase compared to that observed in Figure 5.8b. However, when insulin containing media was decanted after 6 hr and fresh media containing insulin was added for an additional 6 hr there was an average 1.5 fold increase in ALS. This is as was expected in terms of an insulin response which indicated a loss of insulin activity after a 6 hr period due to possible deterioration of the insulin molecule. With a 12 hr insulin treatment and the addition of cAMP for the last 6 hr of this period ALS mRNA levels decreased to levels 20% versus the 12 hr insulin treatment ($p<0.1$). With cAMP treatment of H4IIE cell culture for 30 min followed by 6 hr insulin incubation the accumulation of ALS mRNA decreased 20% versus the 6 hr insulin treatment alone ($p<0.05$). With insulin treatment for 6 hr, media decanted and fresh media without modulator added, ALS mRNA was similar to the values seen for 12 hr

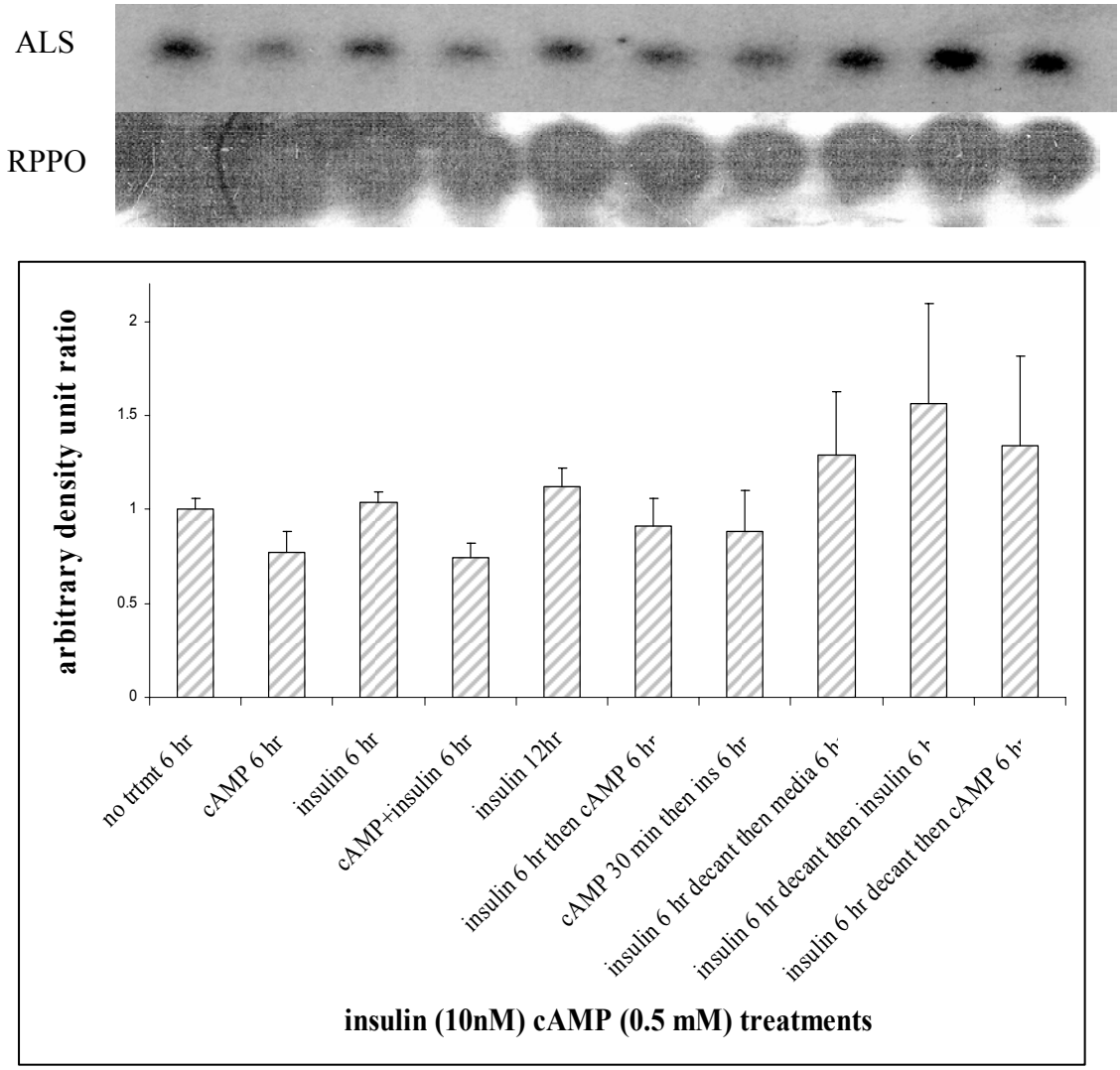

Figure 5.10 The effects of varying cAMP and insulin treatments on ALS mRNA accumulation in H4IIE cells. Over-night serum starved H4IIE cell cultures were treated as seen above and total RNA was isolated and subjected to Northern analysis. The concentrations of insulin and cAMP in all incubations were 10 nM and 0.5 mM, respectively. The above blot (A) is aligned with the correct bar within the graph (B). Densitometry was done using Gel Documentation system and Quantity One software (Bio-Rad). The values represent two sets of independent experiments. P-values of independent experiments with control are $p<0.5$ in all cases measured in comparison to negative control with no treatment.

insulin treatment alone. When the same experiment was done, but cAMP was added in fresh media, after decanting, ALS mRNA levels decreased 15% as compared to experiment where insulin was added for 6 hr, media was decanted and insulin was added for an additional 6 hr. However, there was little difference noted as compared to experiment where insulin was added for 6 hr, media was decanted and fresh media was added for an additional 6 hr.

5.4.4 Effects of PI-3 Kinase and MAP Kinase Pathway Inhibitors on the Regulation of ALS Gene Expression by Metabolic Modulators

It is known that transcriptional effects of insulin are mediated through the PI-3 kinase and MAP kinase pathways (section 2.5.1.2). It is also known that cAMP indirectly interacts with the PI-3 kinase pathway through proteins such as PKC and PP-2A (Begum *et al.,* 1996). As shown in Figures 5.8 and 5.9, ALS gene expression is altered by a number of the metabolic modulators examined, including insulin and cAMP. Wortmannin and PD98059 are inhibitors of the PI-3 kinase and MAP kinase pathways, respectively. Experiments were performed to investigate the effects of wortmannin and PD98059 on the ability of insulin and cAMP to affect ALS gene expression. In all experiments, cells were pre-incubated with wortmannin and PD98059 and this was followed by 6 hr incubation with respective modulators in the continued presence of inhibitor.

The deterioration of insulin was suspected due to mentioned effects noted above (Section 5.4.3). This led us to use a shorter, 6 hr period instead of the prescribed 12 hr period. The results are shown in Figure 5.11a and Figure 5.11b (blots and quantitation bars). cAMP caused little decrease in ALS mRNA levels as was expected (Figure 5.11a; Figure 5.11b, B). This data was in relative agreement with the cAMP response seen in Figure 5.8. H4IIE incubation with insulin also showed little, if any, increase of ALS expression while results using similar time interval and dosage, in Figure 5.8, showed a 1.5-fold increase. These values, again, differ from those determined in Figure 5.8 due to a number of possible factors such as the quality of the modulator used, the quality of the probes used and perhaps the general viability of the H4IIE cells used. The pre-incubation

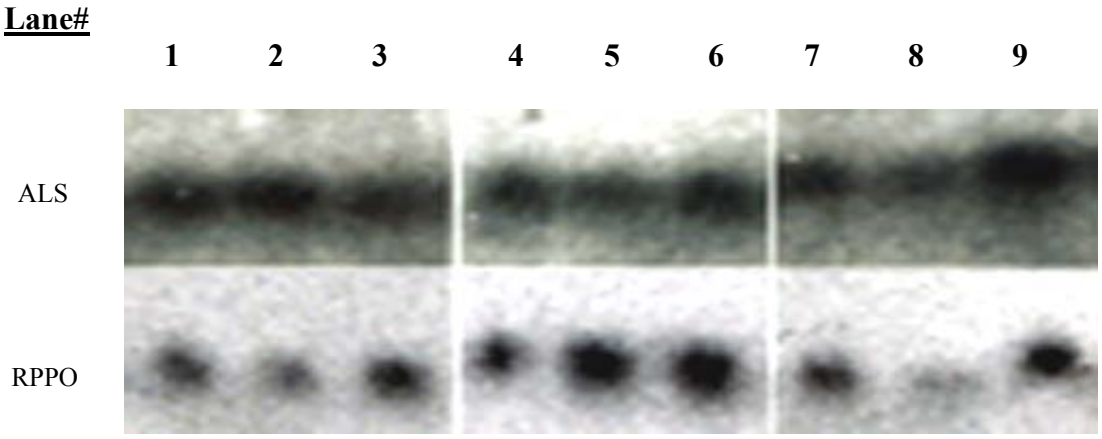

Figure Legend

Lane/treatment #	Cell Treatment and inhibitor
1	No treatment; no inhibitor
2	Insulin; no inhibitor
3	cAMP; no inhibitor
4	No treatment; wortmannin
5	Insulin; wortmannin
6	cAMP; wortmannin
7	No treatment; PD98059
8	Insulin; PD98059
9	cAMP; PD98059

Figure 5.11a The effects of wortmannin and PD98059 on the ability of insulin and cAMP to affect expression. H4IIE cell cultures were pre-treated (treatment 4-9) with inhibitors 30 minutes. Following 30 minute pre-treatment metabolic modulators were added (except for controls) and in all cases cells were incubated for 6 hr. Following treatments media was removed, total RNA was isolated and samples were subjected to Northern analysis with radiolabelled rALS probe. Blot was stripped and re-probed with radiolabelled RPPO probe. Blots shown above represent one of two sets of data analyzed for expression in H4IIE cells.

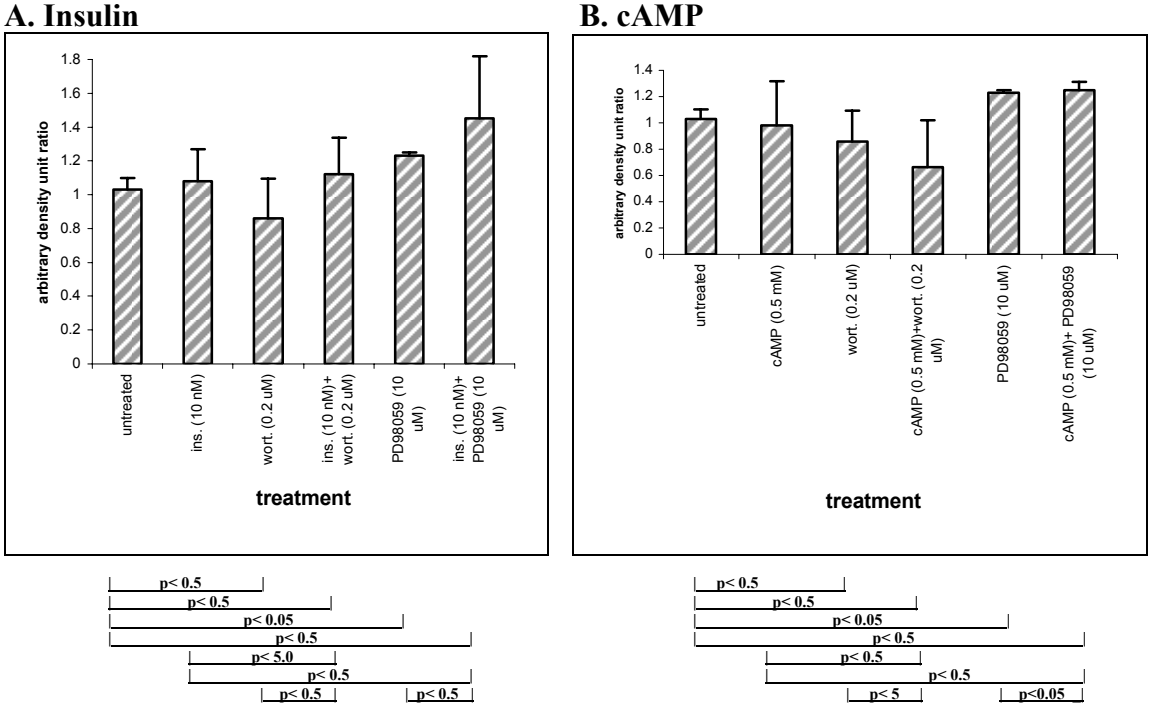

Figure 5.11b The effects of wortmannin and PD98059 on the ability of insulin (A) and cAMP (B) to effect ALS gene expression. Auto-radiographs were analyzed through densitometric analysis using Scion Imaging system. Negative controls were given a value = 1. All ALS mRNA arbitrary density unit ratios were derived using RPPO as a standard for cell expression. Values presented are the averages of two independent experiments (4 values). S.E. is given as an error bar in respective sample plot. P values are indicated below Figures according to the pairs of samples tested. P-values calculated using online T-test provided by SISA (Simple Interactive Statistical Analysis) program.

of wortmannin and PD98059 resulted in a statistically insignificant ($p<0.5$) 17% decrease and a significant 20% increase in ALS gene expression, respectively (Figure 5.11b).

Wortmannin inhibition of the PI-3 kinase pathway and cAMP treatment resulted in statistically insignificant decreases in ALS mRNA to levels 60% of the untreated control and 70% of the cAMP control. A decrease, 70% of the wortmannin control, was also observed (Figure 5.11a; 5.11b, B). Levels of ALS mRNA increased with insulin with levels 10% higher than untreated control, 3% higher than insulin control and 30% higher than the wortmannin control, respectively, all statistically insignificant (Figure 5.11a; 5.11b, A). Inhibition of the MAP kinase pathway in insulin and cAMP signaling caused a significant 15% and 20% increase, respectively, in ALS gene expression when compared to PD98059 control (Figure 5.11a; Figure 5.11b, A and B). PD98059 inhibitor with insulin and cAMP increased ALS gene expression 25% and 20% higher than respective modulator controls, respectively. In comparison to the untreated control, insulin and cAMP (with inhibitor) increased ALS gene expression 30% and 20% (Figure 5.11a, 5.11b). Overall the statistics done on this experiment render these results insignificant and will be discussed as such.

5.4.5 Discussion

The effects of glucose, insulin, GH, dexamethasone and cAMP on ALS mRNA accumulation in H4IIE cells were analyzed as a function of incubation time and their doses. The involvement of the PI-3 kinase and MAP kinase pathways were also investigated with the use of pathway specific inhibitors. In addition, various combinatorial studies were done with insulin and cAMP. Prior to this work, much of the literature on ALS regulation has been derived from *in vivo* studies and *in vitro* primary hepatocyte studies (Guck *et al.,* 1999). Western analysis of ALS from lysate and conditioned media was attempted with a commercially available anti-ALS antibody, but the quality of the antibody was poor resulting in no usable data.

GH has previously been shown to be the most potent hormonal inducer of ALS expression. This has been shown *in vivo* and in primary hepatocyte cultures (Dai *et al.,* 1997, Labarta *et al.,* 1997). ALS is thought to be inducible through a growth hormone-

responsive promoter element in the mouse, sheep and human ALS gene, entitled ALSGAS1 (Suwanichkul *et al.,* 2000, Rhoads *et al.,* 2000). GH activation of ALS expression is through the Jak-Stat pathway (Figure 2.12) which interacts with a diverse number of downstream signaling proteins. The incubation of H4IIE cells with GH (100 nM) for 24 hr showed a 2-fold increase in ALS expression as compared to control. There was a 6-12 hr lag in response and increases in mRNA levels were only seen beyond 12 hr (Figure 5.8a; Figure 5.8b, B). GH dose dependent expression of ALS mRNA, however, showed a 4-fold increase with 500 nM concentration of GH (Figure 5.9a; Figure 5.9b, B). Low responses to GH in time dependent ALS gene expression may be remedied through a higher 500 nM GH concentration as was seen in dosage experiments. Nonetheless, GH has proven to be a relatively strong inducer of ALS mRNA accumulation in the H4IIE cell line.

Although glucose has been implicated in the regulation of expression of many genes (Vaulont *et al.,* 2000), its effects on ALS have not been investigated. As shown in Figures 5.8 and 5.9, glucose up-regulates ALS expression in both a time- and dose-dependent manner. The incubation of H4IIE with glucose resulted in a 3-fold increase in ALS levels as compared to the control. The response was first observed at 6 hr of incubation followed by linear increases with time. A maximal 3-fold increase in dose-dependent ALS expression was observed with glucose (200 mM) as compared to the control. The fact that increases in ALS expression are partially caused by increases in glucose levels may partially negate the possibility that enhanced cell growth during time course experiments were responsible for increased ALS expression. However, further work will be required to be conclusive. Nonetheless, our data suggests that glucose levels positively regulate ALS gene expression in H4IIE cells.

Dexamethasone has been found to be a negative regulator of ALS gene expression at the transcriptional level in primary hepatocytes (Werner 1999). Our time course results indicated a 1.8 fold increase in ALS mRNA accumulation by 12 hr (Figure 5.8a; Figure 5.8b, E). With variation of dexamethasone concentrations there was a positive regulation of ALS mRNA accumulation. This expression reached a 2.5-fold peak at 0.5 μM dexamethasone and then slightly decreased with increased dosages (Figure 5.9a; Figure 5.9b, E). In H4IIE cells, dexamethasone concentration may determine whether the effect

on ALS expression is positive or negative. Further experiments are required to clarify the effect of dexamethasone.

It has been previously observed that cAMP has a negative effect on ALS gene expression in primary hepatocytes. cAMP has been thought to regulate at a post-translational level and has not been shown to regulate at the level of mRNA (Delhanty 1998b). Results from cAMP treated H4IIE cells as a function of time have shown no change in ALS expression in the first 6 hr. Expression increases after 12 hr to a maximum 2.0-fold at 24hr. It may be that the negative effects of cAMP are lost with increases in time resulting in an ALS mRNA increase as cells resume constitutive ALS production. With increases in cAMP concentrations ALS gene expression significantly decreases to a minimum, 64% of the negative control, indicating that H4IIE cell ALS gene expression may be negatively regulated through cAMP (Figure 5.9a; Figure 5.9b, D). Wortmannin had a statistically insignificant negative effect on cAMP negative regulation of ALS gene expression with a level of expression 70% of the wortmannin control, 67% of the cAMP control and 56% of the untreated control (Figure 5.11a; Figure 5.11b, B). From this data, cAMP mediated ALS gene expression and repression may be enhanced by the inhibition of PI-3 kinase activity but it was likely that there was no effect. However, the negative effect of cAMP on ALS gene expression may be dependent on the PI-3 kinase pathway and partially be regulated by it. Further experimentation will be required. Levels of ALS gene expression do not change with the addition of cAMP to PD98059 as compared to PD98059 controls. However, with PD98059 treatment, levels of ALS expression increase significantly versus the negative control. The addition of cAMP did not reverse this effect which may indicate an involvement of the MAP kinase pathway independent of cAMP effects. Further investigation is required to make conclusive arguments about the involvement of cAMP in modulation of ALS gene expression through the PI-3 kinase and MAP kinase pathways.

In primary hepatocytes, insulin induces ALS secretion without any change in ALS mRNA levels. This may suggest a post-translational mechanism of ALS regulation (Dai *et al.,* 1994a). Database searches for proteins which contain consensus sequences for phosphorylation by PKB have shown human ALS to be a likely candidate. A potential phosphorylation of ALS may explain why Dai *et al.,* observed increased ALS secretion

with no change in the accumulation of mRNA. As seen with IGFBP-1, increased phosphorylation may translate to increased secretion. Phosphorylated IGFBP-1 is the predominant form of IGFBP-1 in the bloodstream (Frost *et al.,* 1993). However, our results in rat H4IIE cells clearly indicated a very positive regulation of ALS gene expression in both time course and dose dependency studies. In time course studies the increase was very linear and immediate with a 2-fold increase by 24 hr of H4IIE incubation (Figure 5.8a; Figure 5.8b, F). The dose dependent expression of ALS mRNA was also positive as a 2-fold increase occurred at 10-100nM insulin (Figure 5.9a; Figure 5.9b, C).

The importance of insulin in the regulation of ALS expression was shown in STZ-induced type I diabetic rats. These rats developed hyperglycemia and hypo-insulinemia (not assayed for) which we found was accompanied by a large decrease in ALS gene expression in the liver (Figure 5.1). Decreased ALS in circulation in such conditions may result in decreased ternary IGF-1 complex formation. This translates to possibilities that IGF-1 may be more active in IGF-1 sensitive tissues as well as more transportable across epithelial barriers. However, less ALS in circulation may also be indicative of a more rapid IGF-1 degradation in the circulatory system. The two possibilities may, however, balance each other out resulting in little or no change in IGF-1 activity. Further experimentation is required to elucidate the effects of diabetes on IGF complex formation and downstream results.

Results in HepG2-PKB-CA displayed a 1.2-fold increase in ALS mRNA accumulation (Figure 5.2) indicating a possible importance for the PI-3 kinase pathway in activation of ALS expression. The inhibition of the PI-3 kinase and MAP kinase pathways using wortmannin and PD98059 treatment caused a statistically insignificant 17% decrease and 20% increase in ALS gene expression compared to the negative controls, respectively (Figure 5.11a; Figure 5.11b, A). It was observed that insulin mediated positive regulation of ALS gene expression occurred independent of the PI-3 kinase pathway. Addition of insulin to wortmannin treated cells appeared to reverse the negative effect of wortmannin and increased insulin levels to that of the insulin control, although statistically insignificant. This was surprising as PKB, a downstream component of the wortmannin inhibited PI-3 kinase pathway, was shown to be required for the

positive regulatory activity of insulin on ALS gene expression (Figure 5.2). The effects of insulin on PD98059 inhibited H4IIE cells appeared to be additive as the resulting ALS gene expression was significantly larger than insulin control and PD98059 controls. However this was also shown to be statistically insignificant. It appears, therefore, that in H4IIE cells insulin effects on ALS gene expression may act independent of the PI-3 kinase pathway but may be controlled through the MAP kinase pathway. There may be complicated crosstalk between these two pathways whose interactions result in a positive regulation of ALS. However, due to the lack of statistical significance this will have to be investigated further.

The results in our investigation of the effects of varying cAMP and insulin treatments on the expression of ALS were largely statistically insignificant. However, in H4IIE cells a decrease in ALS gene expression with 6 hr cAMP incubation was observed and insulin increased expression at 6 hr incubation with further increases seen at 12 hr. Interestingly, the H4IIE co-incubation with insulin and cAMP appeared to have no effect of insulin but rather an increased negative activity in comparison to the cAMP 6 hr samples. The activation of cAMP activities in the cell may have inhibited the activity of insulin. This is possibly due to activities of phosphatases such as protein phosphatase 2A interaction with PKC and resulting affects on the PI-3 kinase pathway (Begum *et al.,* 1996, Ugi *et al.,* 2004). As little as 30 min exposure to cAMP may be required for inactivation of the insulin cascade responsible for the upregulation of ALS. This will need to be further investigated to make valid conclusions.

6.0 CONCLUSIONS AND FUTURE STUDIES

Our interest in ALS and IGFBP-1 expression arose from observations that suggested the involvement of metabolic modulators in their regulation. Insulin, for example, has been shown to regulate both ALS and IGFBP-1 expression in hepatic cells. Therefore, the fundamental reason for performing the work described in this thesis was to elucidate the regulation of ALS and IGFBP-1 by metabolic modulators and further the understanding of the role of nutritional/hormonal status on the regulation of IGF-1 bioactivity and address the hypotheses made in the introduction section of this thesis (Section 1.0).

The expression and secretion of IGFBP-1 in H4IIE cells is positively regulated by glucose and GH and negatively regulated by insulin. HepG2-PKB-CA cells showed a significant decrease in IGFBP-1 expression as compared to parental HepG2 cells implicating PKB in repression of IGFBP-1. PKB is activated through the PI-3 kinase pathway and may also interact with the MAP kinase pathway. Inhibition of these pathways and addition of GH, glucose or insulin showed GH to act independent of these pathways while glucose and insulin may be partially dependent. With the inhibition of the PI-3 kinase pathway the normally negative effects of insulin were reversed with an increased IGFBP-1 expression. However, comparison to the wortmannin control shows IGFBP-1 levels to be much smaller with the addition of insulin and experiments using PD98059, a MAP kinase inhibitor, indicated insulin activity to increase in strength with inhibited MAP kinase pathway activity. Therefore, IGFBP-1 regulation may be negatively regulated by insulin through the PI-3 kinase pathway but this does not appear to be likely as the statistics are not in support of this idea. There does however seem to be interaction between these two pathways in IGFBP-1 secretion. In the future I would like to also look at IGFBP-1 phosphorylation and determine what roles, if any, the PI-3 kinase and MAP kinase pathways have in this capacity. Inhibition of PI-3 kinase pathway resulted in an additive glucose effect. Inhibition allowed a large glucose mediated increase in IGFBP-1 expression. Future studies will be required to better characterize the regulation of IGFBP-1 by GH, glucose and insulin. Some research has suggested mTOR involvement in insulin regulation of IGFBP-1 regulation. Inhibitor studies utilizing

rapamycin could be utilized for investigation of mTOR involvement in IGFBP-1 regulation. Important future research would also include the analysis of serum IGFBP-1 isolated from STZ-treated diabetes-induced rats with the hypothesis that serum IGFBP-1 will increase due to increased glucose and decreased insulin in circulation. Analysis may also be completed for the complexation of IGFBP-1 with IGF-1 within serum fractions of STZ-induced diabetic rats.

The expression of ALS mRNA in H4IIE cells is positively regulated by GH, glucose, dexamethasone and insulin and negatively regulated by cAMP. HepG2-PKB-CA cells showed a significant increase in ALS gene expression as compared to parental HepG2 cells. The addition of insulin to these cell types increased expression of ALS equally. This may indicate an activity independent of PKB and the PI-3 kinase pathway. As mentioned previously, PKB is activated through the PI-3 kinase pathway, but may interact with the MAP kinase pathway. Inhibition of the PI-3 kinase and MAP kinase pathways and addition of insulin was performed. These results were insignificant and may need to be repeated to increase the sample size and obtain meaningful data on which to base a conclusion. The effect of insulin on ALS expression may act entirely or partially through the MAP kinase pathway and not the PI-3 kinase pathway as thought. Further studies will be required to provide the appropriate data.

cAMP activity was also assessed in relation to pathway inhibition. Although investigated the data gathered was insignificant. The interactions between cAMP and insulin in terms of their separate effects on ALS gene expression were also investigated in combination studies. These results were also shown to be statistically insignificant. The interactions between these two hormones are important as they serve to represent the metabolic status of an individual and determine the expression levels of ALS mRNA which in turn determines the amount of stable IGF available in serum. These experiments should be repeated a number of times to increase the sample size and provide more reliable and statistically conclusive data. Future experimentation should include factorial studies which will give further information on the combined activities of cAMP and insulin.

The results from rat studies have clearly shown a decreased ALS gene expression in STZ-induced diabetic rats with decreased insulin release. The addition of insulin to

these STZ treated rats returned ALS levels to those of untreated controls. Further research could utilize this *in vivo* STZ-induced diabetic rat model to investigate primary hepatocytes for ALS mRNA levels and serum samples for measure of glucagon and insulin levels. An overall view of the expression of ALS in relation to hormonal levels can be done through experiments designed to look at complex formation using serum samples and methods to isolate the IGF-1 complex and respective components (ALS, IGF-1 and IGFBP-3 or -5). This could be done in isolation with normal conditions and abnormal conditions such as those associated with disease. Of personal interest is the relationship between disease conditions such as diabetes and cancer (Regimbeau *et al.,* 2004, Giovannucci 2001). A relationship between IGF and tumour formation has been shown in literature (Manousos *et al.,* 1999, Khandwala *et al.,* 2000, Macaulay 1991). The availability of IGF is dependent on ALS and complex formation which stabilizes IGF in circulation, allowing an available supply of IGF. The effects of STZ-induced diabetes include a significant decrease in ALS in rats. This could translate to less IGF complexation in circulation and either more available free IGF to interact with IGF receptive tissues or a large decrease in available IGF-1 due to degradation. It must be determined which occurs, as transformed cells may be especially sensitive to such rises in IGF-1 levels. Another issue is Type 2 diabetes which is accompanied by hyperinsulinemia. Increased levels of insulin may cause large increases in ALS which may exist in circulation uncomplexed due to limited availability of IGF-1-IGFBP-3 complexes. However, increased levels of insulin would mean a large decrease in IGFBP-1 which could also translate to increased free IGF-1 expression. This also will need to be investigated to clarify the roles of the IGF system in disease.

In our preliminary investigations of potentially PKB phosphorylated proteins we noted a PKB consensus sequence for phosphorylation within ALS. Although our data exists based on ALS gene expression and not ALS protein it is very desirable to investigate the possibility of ALS phosphorylation by PKB and the function of this phosphorylation. This could initially be investigated through *in vitro* studies to see whether it occurs at all and later, in cell culture studies, to investigate whether this occurs in cells. However, available anti-ALS antibody did not work well against H4IIE conditioned media and lysate samples.

In conclusion, both IGFBP-1 and ALS are significantly regulated in cells of hepatic origin through a number of metabolic hormones. There is strong correlation between metabolic status and the levels of ALS and IGFBP-1 expression. The use of the newly available rALS probes made visualization of ALS gene expression possible, although very difficult, within H4IIE and HepG2 cells. It was also shown that PKB levels are involved in both IGFBP-1 secretion and ALS gene expression but the involvement is unclear at this point as inhibitor studies did not give any clear and undeniable evidence as to the involvements of the PI-3 kinase and MAP kinase pathways in PKB effects. In STZ-induced diabetic rats the expression of ALS is decreased. This may indicate the possibility of downstream physiological effects involving altered IGF system functions. The potential of this research area is very promising and exciting in terms of research into disease etiology and prevention as well as continuing the endeavor to increase our understanding of animal biology and biochemistry.

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